STUDIES IN THE CENTRE OF GRAVITY CHANGES

No. 5

ON THE "BLOOD SHIFT" PRODUCED BY GRADED COMPRESSION OF THE THIGHS.

by

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By means of a method already published (Cotton, 1931, 1932) for recording graphically changes in the Centre of Gravity of the body it has been possible to study the displacement of mass within the resting body under different conditions.

It is the purpose of the present paper to enquire into the mass displacement produced by compression of the thighs, which effect is presumably due in the main to the restriction of venous outflow from the lower limbs, while allowing the entry of arterial blood.

METHOD.

To begin with the subject was approximately balanced upon the Centre of Gravity table, equilibrium being completed by allowing a circular spring balance to support the slightly heavier end (the cranial end). Any shift of mass in the cranio-caudal direction was recorded graphically by a writing arm attached to the index of the spring balance. A rise in the writing lever thus means a shift of mass away from the cranial end.

Upon reclining on the Centre of Gravity table, a compression bag was wound snugly round each thigh. These bags were similar in construction to the ordinary sphygmomanometer bags, but the rubber air reservoirs were made long enough to encircle completely the thigh of a fairly large man. They were about five inches in width.

After initial equilibrium was attained, the desired degree of compression was applied by sudden inflation from a reservoir of air under a surplus head of pressure. The degree of compression was read by a Tycos manometer connected by a T-piece to the rubber bags.

Preliminary experiments showed that low compression always produced an accumulation of mass in the lower limbs, but that when the pressure was excessive the reverse was the case. Presumably in the latter case the rate of onset of pressure was such that the amount of blood dammed back in the venous and capillary reservoirs beyond the compression bags was more than balanced by the diminution in volume of that zone of the thighs beneath these bags.

It was found, moreover, that the maximum mass shift was produced by increasing the compression in stages, so that after some further preliminary work a

uniform procedure was adopted of instituting a series of stages in the compression. The stages finally adopted were 60, 90, 120, and 140 mm. of Hg. In each stage the recording lever rose to a plateau, the establishment of which was variable in time in the different subjects. The time of application of any given pressure was therefore only so long as to attain a definite plateau. The average total time of compression from first to last was of the order of four minutes.

The earlier results showed that the absolute maximum of blood shift could not in general be obtained by these four stages, but a pressure limit had to be struck that would be bearable by a reasonable number of subjects, and it was found in practice that higher compressions than 140 mm. produced sufficient discomfort

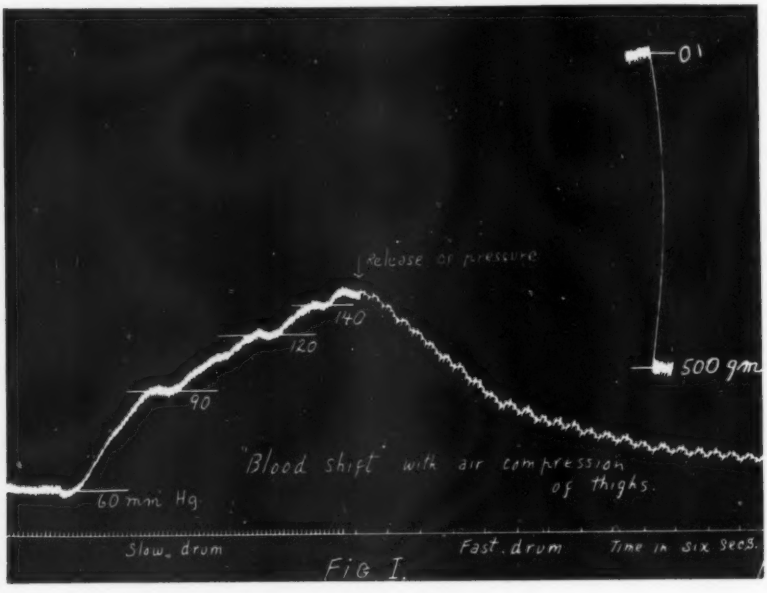

FIG I.

to render them unsuitable for the routine procedure. It is estimated that rather more than 90 per cent. of the maximum attainable mass shift is obtained in this way.

To conclude the experiment, decompression was established suddenly in order to study the rate of unrestricted flow out of the occluded limbs. The compression phase of the record was run with a slow drum, while the decompression phase was run with a fast drum.

Fig. 1 illustrates the course of the stages in the compression phase and also the decompression phase. The calibration arc in the latter figure is obtained by loading the cranial end of the board 120 cm. from the fulcrum with a weight of 500 gm.

As explained in the previous studies (Cotton, 1931), the recording index is caused to move up and down not only with each respiration but also with each heart beat. The amplitude of these oscillations is greatly reduced by attaching to the caudal end of the board a horizontal circular disc about nine inches in diameter which is kept immersed in a viscous oil. The time lag thus introduced in following mass displacements is readily determined by placing a load on the balance board

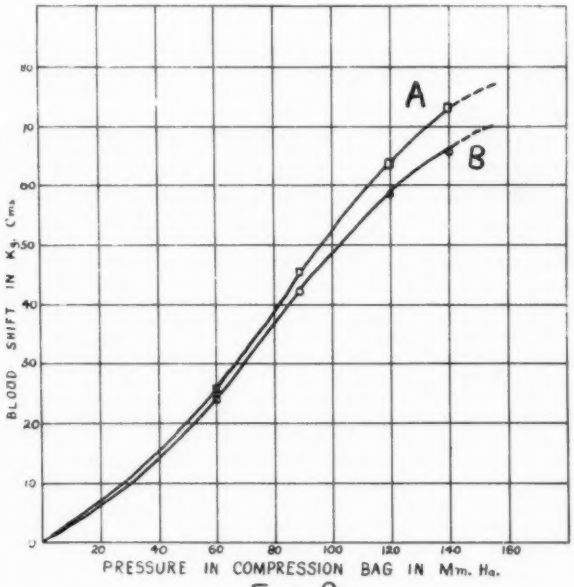

FIG. 2

A. Group 1. B. Group 2.

and obtaining a falling curve with the moving drum. The load used for the determination was 500 gm., which produces a fall of the writing lever of much the same order as the average for the series of subjects concerned. The data obtained from this curve were used to correct the time relations as measured for the recovery process.

RESULTS.

A. The Mass Shift.

Any given instance of mass shift was expressed as so many kg. cm. by multiplying the total change in the supporting force in kg. as obtained from the record, by the distance from the fulcrum to the line of action of the spring balance, namely 120 cm.

The results of the measurements of mass shift were arranged into two groups by the arbitrary procedure of taking all the subjects with odd numbers in one series and all the even numbers in a second series. The agreement between the two groups affords some measure of the stability of the averaged results thus obtained. There were in all twenty-six subjects. The essential results are expressed graphically in Fig. 2.

The upper trends of the curves are dotted to indicate the probable extrapolations as indicated by a few results only.

As explained above it was judged inadvisable to use higher pressures in the routine experiment for fear that the discomfort so produced would jeopardize the recovery curve of the decompression phase.

Although the extent of the data (twenty-six subjects) is not sufficient for serious statistical treatment, the usual computations were made so as to give some general idea of the spread of the individual results. With this reservation the values so obtained may be set out as follows:

Mass displacement in kg. cm. due to progressive thigh compression up to 140 mm. Hg.

Mean	69·5 kg. cm.
Median	69·0 „ „
Lower Quartile	54·0 „ „
Upper Quartile	83·0 „ „
P.E.	14·5 „ „
S.D.	21·5 „ „
Coefficient of Variation	0·31

It will be seen from this that the individual data are very variable, in fact, of the order of twice as variable as the body weight of the subjects.

Since it is to be expected that the mass displacements would show a positive correlation with body weight this coefficient was computed and found to be $0 \cdot 59 \pm 0 \cdot 08$. While this represents undoubted correlation the relationship is not a very close one, being about the same degree as the relationship between body weight and height.

Since, further, it is reasonable to suppose that the blood pressure of the subject might well be a factor in the extent of the mass displacement, the systolic and diastolic pressures were measured as a routine procedure before the commencement of each experiment. This permitted of calculating the coefficient of correlation between systolic pressure and mass displacement, which was found to be $-0 \cdot 22 \pm 0 \cdot 13$. This, however, is so low as to do nothing more than indicate that an inverse relationship of a low order may exist between these quantities.

When, however, the mass shift is expressed in terms of the body weight of the subject, e.g. in gram centimetres per kilogram of body weight, a more definite relationship appears. Thus the correlation coefficient between systolic blood pressure and mass shift in gm. cm. per kg. is found to be $-0 \cdot 42 \pm 0 \cdot 11$, which is sufficiently reliable to indicate that increase of systolic blood pressure is a considerable factor in decreasing the extent of the mass shift under the conditions of the experiment.

No profitable result was obtained by the consideration of body height.

The statistical quantities computed when the mass shift is expressed in terms of body weight are as follows:

Gram centimetres of mass displacement per kg. of body weight.

Mean	1025 gm. cm.
Median	1030 „ „
Lower Quartile	875 „ „
Upper Quartile	1190 „ „
P.E.	157 „ „
S.D.	202 „ „
Coefficient of Variation	0·20

B. The Time Relations of the Recovery Phase.

When decompression occurs, recovery commences quickly, but since final equilibrium is established but very gradually, it is not feasible to measure the precise time when it is completed. It was decided therefore to average the results by determining in the case of each curve the time for 1/4, 1/2, 3/4, and 7/8 of the total recovery. The data thus obtained were arranged as before in two groups (odds and evens) in the arbitrary order of date sequence, to test the stability of the average values.

These results are shown in Fig. 3.

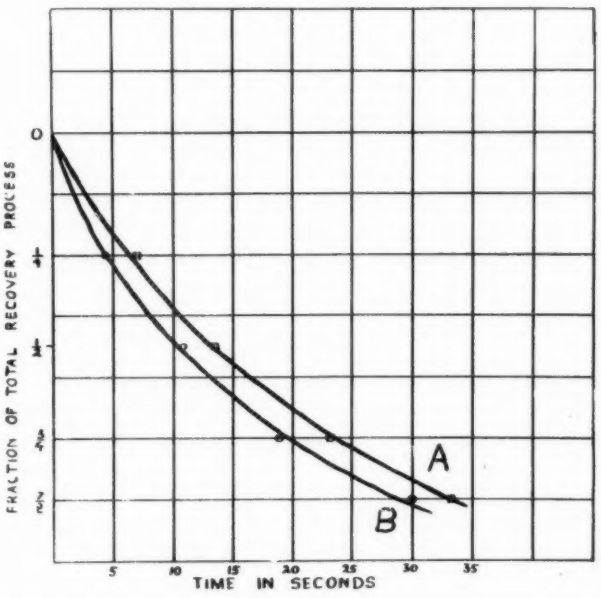

FIG. 3

A. Group 1. B. Group 2.

As in the previous instance where the group averages were compared, the results are of the same order of magnitude but not in so close agreement. Since, however, in both these graphical comparisons the constituent points of each curve are in good agreement, quite a close relative consistency obtains within each group of averages concerned.

DISCUSSION.

The nature of the mass displacement is clear enough from a consideration of the time relations of the recovery process. Since this is largely completed in something of the order of half a minute, most of the effect must have been produced by the damming back of the blood itself in distended capillaries and veins, and very little scope remains for exudation of lymph to play a part. For this reason this mass displaced is referred to as the "blood shift" to denote at least the large fraction of the total change.

As in the case of the previous study in centre of gravity changes (Cotton, 1932) it is possible to obtain from this "blood shift" value (which has so far been expressed in units of kg. cm.) some concept as to the extent of increased volume of the vascular reservoirs in the constricted limbs. It will doubtless be readily conceded that it is scarcely possible for the mass of blood concerned in the shift to have been displaced on an average of more than 80 cm., for this would imply in the average subject a shift of blood from a mean position rather higher than the nipples to a mean position a little below the patella.

If therefore 80 cm. is a maximum value for the distance factor in the average "blood shift", a minimum value for the quantity of blood involved must be $69 \cdot 5/80$ kg. $= 870$ gm.

Hence we may safely conclude (especially when it is recognized that the experimental procedure does not produce the full extent of the possible blood shift) that by placing an appropriate compression zone round the thighs, the vascular reservoir of the limbs below this can be made to accommodate on the average *at least 870 gm. or nearly two pounds of blood more than the normal amount.*

SUMMARY.

By means of the Centre of Gravity table a study has been made of the supranormal amount of blood that can be accommodated by the vascular reservoirs of the lower limbs by damming the venous outflow from the thighs with pressure bandages.

The time relations indicate that exudation of lymph into the tissues can play but a very minor part in the change produced.

Data from twenty-six subjects indicate that a minimum value for the average additional amount of blood so accommodated is rather more than 800 gm. Upon decompression approximately 90 per cent. of the blood shift is restored in about half a minute.

ACKNOWLEDGMENT.

I wish to express my thanks to Professor Davies in whose laboratory the work was done.

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THE VENOM OF THE PLATYPUS (*ORNITHORHYNCHUS ANATIMUS*)

by

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The male platypus is provided with a sharp canalized spur on the inside of each hind limb near the ankle. Through the opening of the canal near the tip of the spur the secretion of a crural gland is extruded when the spur is erected to the striking position. Each gland is placed high on the dorsal aspect of the thigh and connected to the canal in the spur by a duct 5 cm. in length. Early opinions were divided as to the function of these structures, but the view is now generally held that the apparatus is "an offensive and defensive weapon" (Wood Jones, 1923). Burrell (1927) inclines to the belief that the spurs, as first suggested by Home (1802) are also used in clasping the female during copulation. However this may be, it is well established that men and dogs (*vide* Anderson Stuart, 1894) have many times exhibited a characteristic train of symptoms indicative of envenomation following wounds caused by the male platypus.

A number of cases of wounds in man followed by severe pain, symptoms of shock, and swelling of the region of the wound and of neighbouring parts have been described. Since Tidswell's survey in 1906, two such were recorded by Kent Hughes (1931) and another with almost identical symptoms has come to my notice. It is also well known that wounds by the spurs of platypus are not invariably attended by symptoms, either because of seasonal variation in the venom, as suggested by Martin and Tidswell, or because no venom happened to be available at the moment when the strike occurred.

Little is known of the toxicology of the venom, apart from the work of Martin and Tidswell (1895) who recorded a few observations on the effects of the injection of the venom into rabbits.

The subcutaneous injection of 50 mg. of venom in an average-sized animal was followed by loss of appetite and and hebetude for 48 hours. Extensive oedema spread from the site of injection, reached its height after 24 hours and subsided on the fourth day, by which time recovery was complete.

The intravenous injection of 60 mg. in a rabbit weighing about 1·5 kg. caused death in 90 seconds from intravascular coagulation. In another experiment 400 mg. of dried alcohol

precipitate were injected very slowly into a vein in a rabbit of about the same weight and caused a profound fall of blood pressure. A second similar dose half an hour later was followed by sudden death with universal intravascular coagulation. In a fourth experiment a fall of blood pressure was caused by 20 mg. of venom injected intravenously. A second injection 90 seconds after the first caused a further fall of blood pressure, and death occurred in 2½ minutes. The blood in this case was fluid with a prolonged coagulation time (12 instead of the normal 3 or 4 minutes).

These observations demonstrated the presence of a thrombin in the venom and showed that its chief obvious action was to cause a profound fall of blood pressure.

The gift of a considerable amount of the dried venom of the platypus by Sir Charles Martin has enabled us to extend these early observations upon the toxic effects of this venom. This sample consisted of yellowish pseudo-crystals resembling dried snake venom, which lost no additional water upon further drying *in vacuo*. It was readily and almost completely soluble in physiological saline solution, yielding, when dissolved in a concentration of 50 mg. per c.c., a turbid solution from which a small amount of insoluble residue settled on standing.

The Results of Intravenous Injection in Non-anaesthetized Rabbits.

With this sample of venom we were not able to demonstrate any coagulant effect in doses of 30 and 60 mg. per kg. Rabbits weighing 1·64 and 1·68 kg. which received the venom in these doses in concentrations of 20 and 50 mg. per c.c. exhibited identical symptoms. They rapidly became uneasy and developed severe dyspnoea which lasted for 7 or 8 minutes. There were repeated swallowing movements, and the bladder was evacuated. Recovery was complete in between 12 and 13 minutes. There was some delay in the coagulation time of blood taken from an ear vein within an hour after recovery. A sample from the rabbit which had the smaller dose clotted in 13 minutes.

Miss Williams made some observations for us upon the coagulant action of the venom *in vitro* upon the citrated plasma of man and guineapig. In human citrated plasma (0·2 c.c.) a soft clot was produced by 2·0 mg. of the venom (in 0·1 c.c. saline) in 50 minutes at 21°C., but no clotting occurred during 3 hours in further tubes to which 0·1 c.c. of solutions containing descending tenfold dilutions of venom were added. In guineapig citrated plasma (0·2 c.c.) 2·0 mg. of venom in 0·1 c.c. saline caused a soft clot in 9 minutes at 21°C. and 0·2 mg. of venom caused a soft clot in 40 minutes. No clotting occurred during 3 hours with the addition of descending tenfold dilutions of venom.

The coagulant activity of this old dried venom may have been diminished by denaturation since it was probably at least 30 years old. Alternatively, since it was a pooled specimen, its coagulant action may have been reduced by admixture of venom in which, owing to seasonal variation, this factor was absent or present only in small amount.

A fresh sample of venom was obtained on September 26th, 1935, by aspiration from the ducts of a large male platypus recently dead. This was dried and

weighed. When redissolved in saline, in a concentration of 50 mg. per c.c. on October 4th, it failed to cause definite intravascular coagulation when injected intravenously in a dose of 50 mg. per kg. in a rabbit weighing 1·44 kg. which was fully anaesthetized with ether. Blood taken from an ear vein half an hour after the injection yielded a soft clot in 4½ minutes and was clotted solidly in 16 minutes. Two minute particles of whipped-out fibrin were found in the right heart when this animal died suddenly 2¼ hours after the injection. This sample of venom was probably somewhat more potent in respect to coagulant action than our old dried specimen, which contained only traces of thrombin.

The Results of Subcutaneous Injection.

The subcutaneous injection of the old sample caused effects similar to those produced in Martin and Tidswell's experiment.

A rabbit weighing 2·1 kg. received 40 mg. under the skin of the abdominal wall. There was extensive haemorrhagic oedema spreading over an area of about 18 sq. cm. A piece of skin and subcutaneous tissue was removed for section after 24 hours and showed the nature of the oedema, a good deal of blood-stained fluid and some free blood escaping. The animal was quiet for a day or so but survived without any serious symptoms.

A guineapig weighing 360 gm. received 100 mg. of venom under the skin of the shaved abdominal wall. The local lesion produced was a very extensive oedema spreading over about half the ventral surface of the body. The removal of a piece of skin and oedematous subcutaneous tissue for section after 24 hours liberated much oedematous fluid containing a few red blood cells. Microscopic examination of the sections showed intense leucocytic infiltration. The animal was sick for 2 or 3 days but continued to eat moderately well and survived without obvious suppuration at the site of the injection.

This sample of venom had not lost its highly irritant action on the subcutaneous tissues, the rapid appearance of haemorrhagic oedema indicating the presence of a cytolysin with a destructive action upon vascular endothelium. The leucocytic infiltration in the guineapig suggested that the sample was not sterile and, although no further investigation was carried out to check this point, we used in our experiments only freshly made-up solutions of venom. There was not the slightest hint that the injections resulted in pain. The whole of the local lesion appeared to be anaesthetic at the time when pieces were removed for section.

The Haemolytic Action of the Venom.

Miss Williams has tested the haemolytic power of the venom *in vitro* using human and guineapig erythrocytes. The haemolytic system had a volume of 0·3 c.c. (0·1 c.c. of 3 p.c. suspension of corpuscles, 0·1 c.c. of saline and 0·1 c.c. containing doubling dilutions of the venom), 2·0 mg., 1·0 mg., 0·5 mg., and so forth. The tubes were observed at 37° C. and, owing to the possibility that the venom was not sterile, no attention was paid to readings later than four hours. Human red blood cells were not haemolyzed within this time by any of the concentrations of venom used. Guineapig erythrocytes were lysed by 2 mg. in ¾ hour, by 1·0 mg. in

1 hour, by 0·5 mg. in 1½ hours, by 0·125 mg. in 2½ hours, and by 0·25 mg. in 3½ hours. Since guineapig and human corpuscles are both extremely susceptible to haemolysis by venoms these results serve to emphasize the extremely feeble haemolytic power of the venom.

The Results of Intravenous Injection in Anaesthetized Animals.

In rabbits anaesthetized with Dial 0·05 gm. per kg. with additional ether for the necessary operative procedures, platypus venom injected intravenously regularly caused a profound fall of blood pressure with doses varying from 5·0 to 30 mg. per. kg. Figure 1 shows the response in a rabbit weighing 1·68 mg. to a dose of approximately 30 mg. of platypus venom per kg. There was a rapid fall of blood pressure in the first minute after the injection, and the level continued to fall so that within five minutes it had dropped from 65 to 18 mm. Hg. After 34 minutes the blood pressure was still at shock level and the respiration failed. Despite artificial ventilation the animal died in less than 50 minutes. Here the dose of Dial was somewhat too large—0·06 mg. per kg.

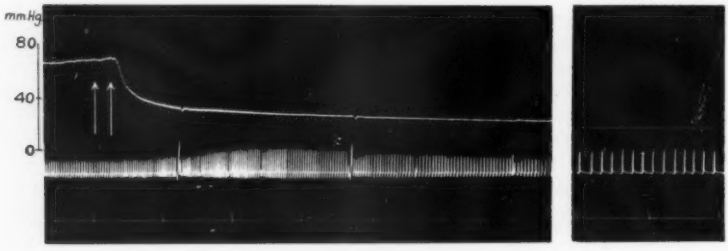

Fig. 1. Record of the blood pressure and respiration of a rabbit weighing 1·68 kg. under Dial and ether anaesthesia. Between the arrows 50 mg. of platypus venom was injected intravenously. The right-hand panel is 34 minutes later. Time in half-minutes.

In this and other experiments anaesthesia appeared to sensitize to the effects of the poison, since non-anaesthetized animals recovered within a quarter of an hour after the exhibition of larger doses. The increase in rate and extent of respiration was shown to be largely a reflex effect due to fall of blood pressure in the sinus caroticus. In numerous experiments we have never succeeded in showing any direct effect of the poison upon respiration, nor was there ever any sign of curarization despite the repeated injection of large doses.

Small doses also caused an immediate fall of blood pressure. For instance, a rabbit weighing 1·88 kg. under ether anaesthesia gave no response to a dose of 4·5 mg., but a definite fall from 66 mm. to 36 mm. Hg. occurred when 9·5 mg. (5 mg. per kg.) was injected two minutes later. The blood pressure had recovered to 50 mm. in half an hour. A dose of 10 mg. per kg. given 1 hour and 58 minutes after the first dose caused a fall of blood pressure from 36 to 22 mm. Hg.

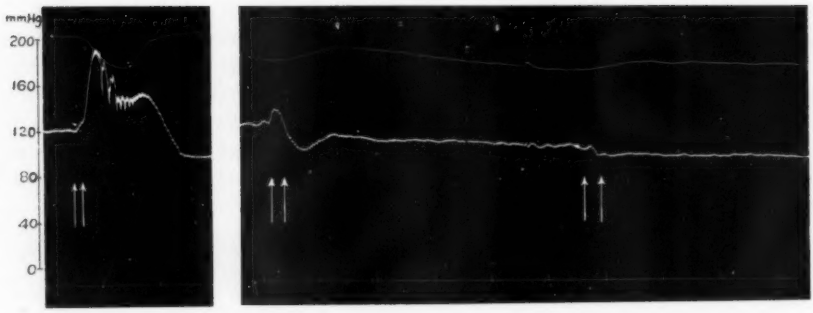

Fig. 2. Record of blood pressure and volume changes in a loop of jejunum of a cat weighing 2·67 kg. under Dial and ether anaesthesia. Between the arrows in the left-hand panel 0·01 mg. adrenaline, and between those in the right-hand panel two doses of 10 mg. of platypus venom were injected. Time in half-minutes.

Fig. 3. Record of blood pressure and volume changes in a hind limb of a cat weighing 3·04 kg. under Dial and ether anaesthesia. Between the arrows 21 mg. of platypus venom were injected intravenously. Time in half-minutes.

Under anaesthesia during the time occupied by our experiments complete recovery even from moderate doses did not occur and animals, during the later stages of the experiment, at the lower levels of blood pressure then prevailing, were less sensitive to the vaso-depressant action of the venom. It is not clear what part anoxaemia of the bulbar centres may play in causing this effect. This action of the venom in the rabbit resembled that of histamine in the cat, the animal under anaesthesia entering a condition closely resembling that of histamine shock.

The fall of blood pressure was shown in plethysmograph experiments to be associated with peripheral vasodilatation. In bowel plethysmograph experiments

the vasodilatation practically coincided with the fall of blood pressure (Fig. 2), but in limb plethysmograph experiments vasodilatation was delayed until the blood pressure had almost reached its lowest level (Fig. 3). This might be interpreted as indicating that vasodilatation in the limb was masked by the fall of general blood pressure, and only became evident when this passive influence had ceased to operate. In any event, it suggests that vasodilatation in the splanchnic bed plays the dominant role in causing fall of blood pressure due to this venom.

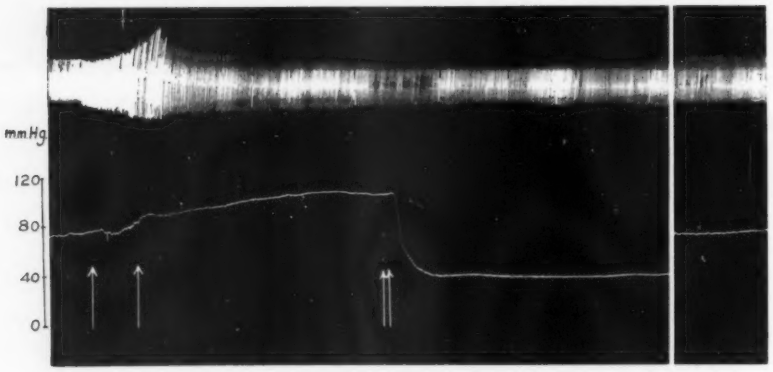

Fig. 4. Blood pressure and tracheal respiratory record from a rabbit weighing 2·1 kg. under ether anaesthesia in which both carotid sinuses had been excluded. In the left-hand panel at the first two arrows the right and left vagi were divided. Between the succeeding arrows 10 mg. of platypus venom was injected intravenously. The right-hand panel is 10 minutes later. The animal was killed when in good condition after a further period of 1 hour had elapsed. Time in half-minutes.

We obtained no definite evidence in plethysmographic experiments of any direct action of the venom upon the heart, nor of any participation of the vagi in the fall of blood pressure. Furthermore, venom does not appear to produce its effect by any direct action upon the carotid sinus mechanism. A fall in blood pressure similar to that produced in the intact rabbit also occurred when both vagi had been divided and both carotid sinuses extirpated (Fig. 4). In this experiment, in which the sinuses were excluded, it will be noted that the fall of blood pressure was not attended by increase in the rate and extent of respiration.

That the presence of an anaesthetic is not essential to the production of the fall of blood pressure by platypus venom was shown by experiments upon the decerebrate preparation. Figure 5 shows the initial response in a decerebrate cat weighing 1·96 kg. to an intravenous injection of 50 mg. of venom. The blood pressure fell from 145 mm. to 68 mm. Hg. in one minute, and after five minutes to 58 mm. Hg. After half an hour the blood pressure had recovered to 88 mm. Hg. A second injection of 50 mg. 1 hour and 20 minutes after the first resulted in a fall

of blood pressure from 64 mm. to 28 mm. Hg. in half a minute, and in 30 minutes the pressure had recovered to 56 mm. Hg. A dose of 16 mg. was now injected, and caused a transitory fall to 40 mm. which in one minute rose to 52 mm. Hg. The vagi were now divided and the blood pressure rose to 65 mm. Hg. A further dose of 16 mg. of venom now caused a fall to 38 mm. Hg., rising in three minutes to 69 mm. In this experiment, though both carotid arteries had been tied, the fall of blood pressure was attended by an increase in the rate and extent of respiration. It should be observed, however, that the dilator response to platypus venom in the decerebrate preparation is less prolonged than in the anaesthetized animal. It is probable, though we have not shown it directly, that the general symptoms which follow intravenous injection of venom in the intact non-anaesthetized animal are due to a transitory but severe fall of blood pressure.

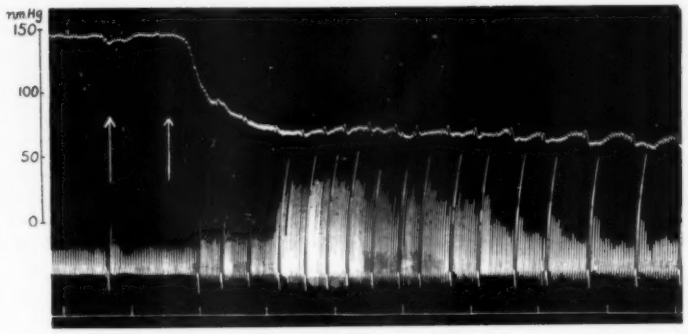

Fig. 5. Record of carotid blood pressure and respiration in a decerebrate cat weighing 1·96 kg. Both carotids were tied and the vagi were intact. Between the arrows 50 mg. of platypus venom were injected intravenously. Time in half-minutes.

In the decapitate preparation, the venom also causes a fall of blood pressure, but the extent of the fall is not so great as in the intact animal, owing to the low initial level of blood pressure.

A cat weighing 1·7 kg. was decapitated under chloroform anaesthesia and, after the anaesthetic had been blown off, a dose of 20 mg. of venom caused a fall from 51 mm. Hg. to 31 mm. in the first minute. Three-quarters of an hour later, the blood pressure had fallen to 29 mm. and a second injection of 20 mg. caused no further fall of blood pressure. Two hours after this injection, a dose of 40 mg. of venom caused a further fall to 17 mm. Hg. A few minutes later, when the pressure was 21 mm. Hg. a dose of 0·1 mg. of acetyl-choline caused a transitory fall to 11 mm. Hg. There was, however, no response to 1·0 c.c. of Padutin and a dose of 0·1 mg. of histamine caused a rise of blood pressure.

Our results in experiments upon decapitate preparations lead us to believe that the vasodepressant action of the venom is mainly peripheral.

In a number of experiments, though we failed to obtain decisive evidence that this poison could completely desensitize the intact animal or the decerebrate or decapitate preparation to subsequent injections of venom, we repeatedly observed some loss of sensitiveness of response to later injections of venom even when allowance was made for lowered levels of mean pressure. The following experiment illustrates this point.

A cat weighing 3·46 kg. was anaesthetized with Dial 0·1 gm. intraperitoneally and additional ether for the operative procedures. A dose of 20 mg. of venom injected intravenously caused the blood pressure to fall from 154 mm. to 84 mm. Hg. After 20 minutes the blood pressure had recovered to 110 mm. Hg. A second dose of 20 mg. caused a fall to 98 mm. Hg. Nine minutes later the blood pressure was 92 mm. Hg., and a dose of 40 mg. caused a rapid fall to 72 mm. Hg.

In experiments with the old sample of venom complete failure to respond was only observed when the mean blood pressure was very low.

In a single experiment in which the effects upon the blood pressure of the old venom and the fresh sample, to which reference has been made, were compared, there was some indication that the persistent vasodepressant action of the old sample in repeated dosage may have been due to the presence in it of a second depressant substance absent from this sample of fresh venom.

In a rabbit weighing 2·6 kg. fully anaesthetized with ether a first intravenous dose of 5·0 mg. per kg. of the fresh venom caused a fall from 80 mm. Hg. to 44 mm. Hg. in 1½ minutes. Fifteen minutes later a dose of 5·0 mg. per kg. of old venom caused a sharp fall from 66 mm. Hg. to 40 mm. Hg. in half a minute. Ten minutes later the blood pressure was 68 mm. Hg., and a third dose of 5·0 mg. per kg. of the fresh venom was without effect, but a fourth similar dose of the old venom five minutes later caused a sharp fall from 64 mm. Hg. to 49 mm. Hg. within half a minute. Further experiments are obviously needed to establish the fact that animals can be completely desensitized to the vasodepressant action of fresh venom.

Our experiments suggest that the venom has a paralysing effect upon the peripheral vasodilator mechanism. In three experiments in cats we attempted to ascertain whether after the repeated administration of platypus venom we could obtain the "ergotoxin reversal" of the response to adrenaline. In none of these after the exhibition first of the venom and then of ergotoxin were we able to obtain the ergotoxin reversal. After ergotoxin, adrenaline in doses of from ·002 to ·02 mg. caused no trace of vasodilatation though its pressor effect had been abolished by the ergotoxin. Figure 6 illustrates a variant of this experiment in which the animal was first poisoned with ergotoxin and the reversal phenomenon demonstrated, then with platypus venom and, finally, tested with adrenaline. The vasodilator response to adrenaline unmasked by the paralysis of the vasoconstrictors by ergotoxin was abolished in all four experiments by the paralysing action of platypus venom. It may, however, be noted that in numerous experiments in which the vasodilator response to platypus venom was found to be impaired, a good vasodilator response could still be obtained with Lacarnol—a preparation containing adenylic acid.

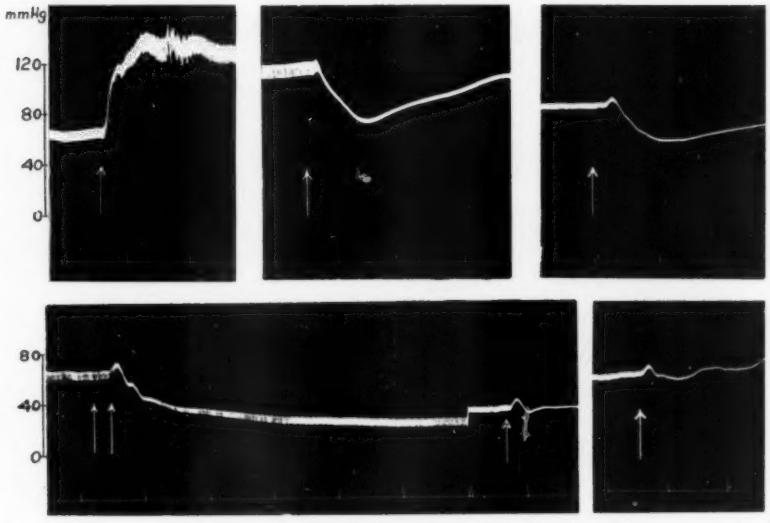

Fig. 6. Record of blood pressure from a cat weighing 1·55 kg. under Dial and ether anaesthesia. The upper left-hand panel shows the response to 0·005 mg. adrenaline at 11.29 a.m. before the administration of ergotoxin. The middle and right panels show the responses to 0·01 mg. adrenaline at 12.5 p.m. and 12.25 p.m. after 6 mg. of ergotoxin administered at 11.41 p.m. Between the arrows at the left of the lower left panel at 12.42 p.m. 18·6 mg. of platypus venom was injected. The right-hand arrow in this panel at 12.52 p.m. and that in the lower right panel at 2.7 p.m. mark the injection of 0·01 mg. of adrenaline. A further dose of 0·65 mg. of ergotoxin was injected at 1.56 p.m. Time in half-minutes.

Experiments upon the Isolated Perfused Rabbit's Ear.

We further studied the peripheral action of this venom upon rabbits' ears perfused through the central artery with buffered saline solution.

We used the Dale-Schuster perfusion pump fed from a two litre reservoir containing oxygenated saline which, together with the pump, was maintained at a temperature of 41°C. The fluid reached the ear at a temperature of 37° to 38°C. The saline used was that recommended by Trevan as modified by Burn (1928) (NaCl 9 gm., KCl 0·42 gm., $CaCl_2$ 0·12 gm., $Na_2B_4O_7$ $\frac{M}{20}$ 3 c.c., and H_3BO_3 $\frac{M}{5}$ 8·5 c.c. per litre). As a cannula a fine hypodermic needle with roughened sides and blunted point was used. The amputated ear was placed on a glass plate and kept warm by a carbon filament lamp below it. Condon's drop recorder was used to record and measure the outflow, which was discarded. To maintain tone in the vessels, adrenaline in constant concentration was added to the perfusion fluid. The arrangements for this purpose are seen in the diagram in Fig. 7. Adrenaline 1:150,000 to 1:200,000 was admitted through a bye pass provided with a valve system (k) of two ball valves placed in parallel and rendered incompetent by reversing one of them, and a buffering reservoir (m) which ensured that the pressure in the bye pass was automatically maintained, having been set just above the level of the mean pressure in the main channel, by raising or lowering the

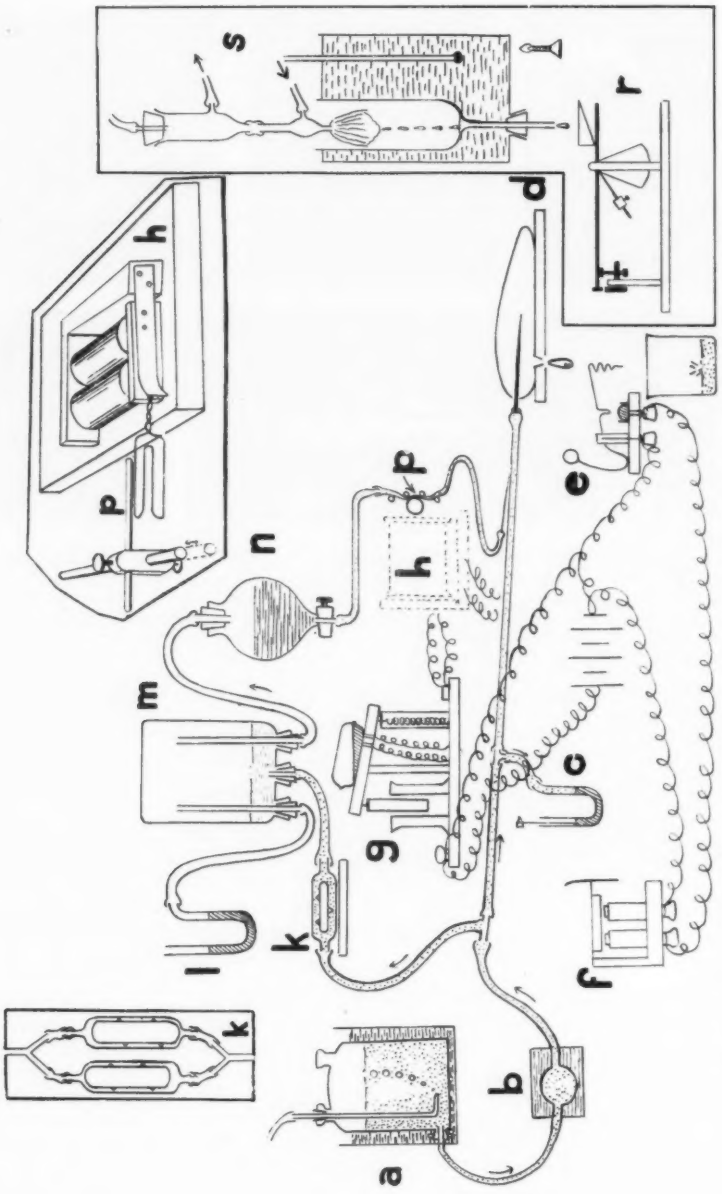

Fig. 7. Diagram of apparatus for perfusion of the rabbit's ear and cat's mesentery with arrangements for maintenance of tone by perfusion with a constant concentration of adrenaline.

level of the buffering reservoir. This could be checked by the readings of the manometers (i) and (e). A second reservoir (n) held the adrenaline solution which was led into the main channel through a hypodermic needle. Between (n) and the needle the adrenaline had to flow through a piece of valve tubing which was threaded through a wire loop connected to the

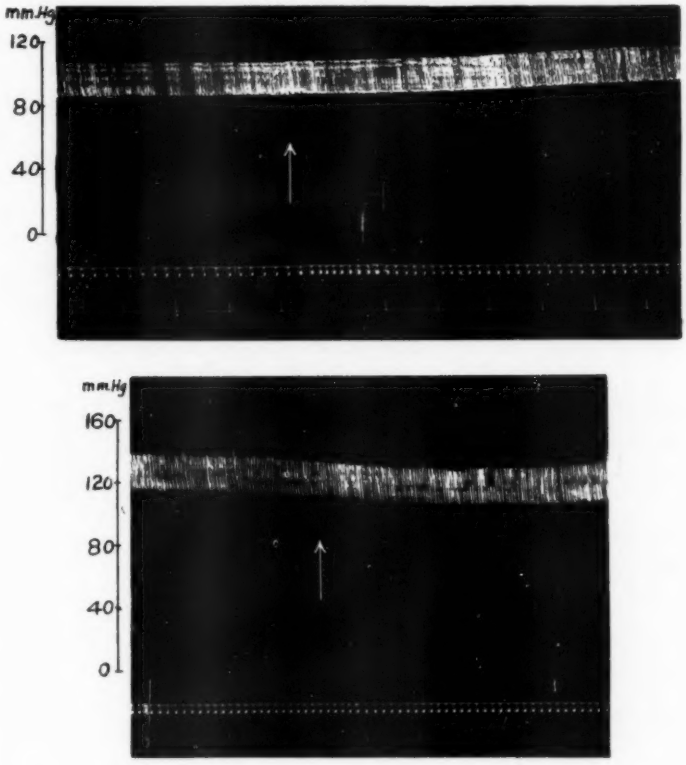

Fig. 8. Record of perfusion pressure and rate of flow in a perfused rabbit's ear in which tone was maintained by continuous perfusion of adrenaline. The arrows mark the injection of 10 mg. of platypus venom. The upper panel shows a vasodilator response to the initial dose of venom. The lower panel shows failure to respond 1½ hours after the first test, the preparation having been desensitized by repeated doses of venom. Time in half-minutes.

iron tongue of an electro magnet (h). An adjustable glass rod (p) was so fixed that the valve tubing was obstructed and no adrenaline could flow unless the magnet were deactivated. Then it acted as a pump forcing a jet of adrenaline into the main channel. This occurred only when a drop of out-flowing saline from the ear depressed the magnetic drop recorder (e); the contact so made activated the magnetic signal (f) on the drum and also a relay (g) which broke the main circuit through the electro magnet (h). Every drop which flowed out from

the preparation therefore caused the entry of a very minute amount of adrenaline into the in-flowing stream. Under these conditions an increased flow through the ear was attended by an increased flow of adrenaline solution and the amount of adrenaline in the perfusion fluid was maintained approximately constant whatever the state of dilatation or constriction of the vessels in the perfused preparation. The buffering system prevented large changes of pressure.

A rabbit's ear perfused in this manner with a uniform concentration of adrenaline gave in successive minutes $8\frac{3}{4}$, $8\frac{1}{2}$, and $8\frac{1}{2}$ drops. During the four successive minutes after the injection of 10 mg. of platypus venom (Fig. 8, upper panel) there were $11\frac{3}{4}$, $11\frac{1}{4}$, 10, and $9\frac{1}{2}$ drops per minute. Ten minutes after the first dose a further 10 mg. of venom were injected, and again vasodilatation occurred. After two further injections a final dose of 10 mg. $1\frac{1}{2}$ hours after the first test dose (Fig. 8, lower panel) gave no appreciable response, the number of drops before being 14 per minute, and after, 13, $13\frac{1}{2}$, 13, and $13\frac{1}{4}$ respectively in successive minutes. Immediately afterwards a good vasodilator response was obtained to a dose of 0·5 c.c. of Lacarnol, namely, 18, 24, 21, and 19 drops in the first four successive minutes.

This preparation can be desensitized by the venom so that, after several injections the ear no longer responds by vasodilation, while it is still capable of active response by vasoconstriction to histamine or adrenaline. We made some further experiments to see whether a simple preparation of small arteries exhibited the same phenomenon.

Perfusion of the Vessels in the Cat's Mesentery.

The preparation was made by anaesthetizing a cat and tying a cannula peripherally into the superior mesenteric artery. The perfusion was now started and the portal vein opened. The mesentery was cut away as close as possible to the bowel and the preparation was freed from the body of the cat and hung up inside a water-jacketed glass vessel (Fig. 7 S). The out low was collected from the outlet tube in the bottom of the vessel, the rate of flow being recorded by means of a Condon's magnetic tipper. The saline used for perfusion and the arrangement of the perfusion was similar to that shown in Figure 7. For the investigation of a dilator substance it was necessary to give the preparation tone by addition of adrenaline to the perfusion fluid. Since the changes of mean perfusion pressure were small we did not in these particular experiments control the inflow of adrenaline through the bye pass, the controlling electro magnet and relay being left out of circuit. When vasodilatation took place the increase in the rate of flow of the perfusion fluid doubtless diminished to some extent the concentration of adrenaline in the perfusion fluid and enhanced the apparent dilatation.

Figure 9 shows an experiment conducted in this way, and illustrates the finding that after repeated doses of venom the vessels become insensitive to its dilator action. In the upper panel is the dilator response to a first injection of 20 mg. of platypus venom, and in the lower is the response to the same dose of venom 1 hour 43 minutes later after four further doses of 20, 20, 40, and 40 mg. of venom had been administered.

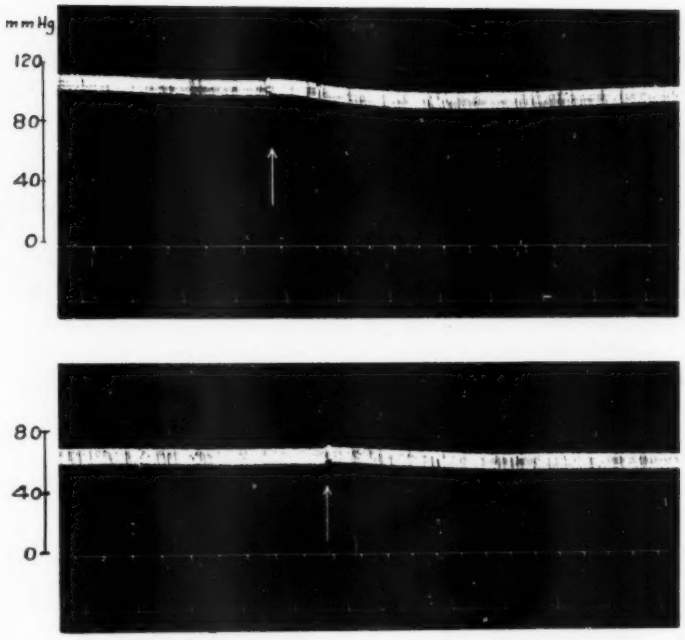

Fig. 9. Record of perfusion pressure and rate of flow in perfused cat's mesentery. The top panel shows the first response to 20 mg. of platypus venom, and the lower panel failure to respond 1 hour and 43 minutes later, after four intermediate doses of 20, 20, 40, and 40 mg. of venom. The outflow was recorded by a Condon's tipper (unit 7·2 c.c.). Time in half-minutes.

In three other experiments in which adrenaline was perfused into the pumping chamber at a constant head of pressure irrespective of changes in the system, the same phenomenon was observed. In these experiments, platypus venom in a dose of 20 mg. caused a substantial fall of perfusion pressure, thus increasing the flow of adrenaline and tending to abolish the dilator effect. The dilator effects observed were, under these conditions, always of short duration. In these experiments varying concentrations of adrenaline were used depending on the rate of inflow of adrenaline into the system. When the adrenaline solution was allowed to flow from a burette connected with a large pressure chamber into the pumping chamber, concentrations of the order of 1:150,000 flowing at a rate of about 1 c.c. per minute were adequate. In the experiment recorded in Figure 9 the rate of inflow of adrenaline from the bye pass through a very fine hypodermic needle was relatively slow, and the concentration necessary was 1:10,000.

In these experiments a number of other substances were tested. Histamine in a dose of 0·1 mg. regularly caused constriction. Acetylcholine 0·1 mg. generally caused dilatation, but in one experiment caused constriction. Lacarnol 0·5 to 1·0 c.c. regularly caused dilatation.

Action of Platypus Venom on Isolated Plain Muscle.

Platypus venom causes contraction of the smooth muscle of the isolated guineapig uterus, and rabbit bowel. In Figure 10 the left-hand panel shows the response of one horn of a virgin guineapig uterus to 0·01 mg. of histamine, and the right panel shows the delayed but maximal response to 10 mg. of platypus venom. The delay in the response and the differing nature of the response to smaller doses of venom (Fig. 11) excludes the possibility that contraction of the smooth muscle is due to the presence of a histamine-like contaminant in the venom. In Figure 11 the response to 2·0 mg. of platypus venom which, in a bath of 110 c.c. capacity, is somewhere near the threshold, shows a prolonged delay, and is quite different from the immediate response of the matching doses of 0·002 mg. of histamine. Figure 12 shows the effect of a minimal effective dose of 2·5 mg. and of twice this amount upon a short segment of rabbit's bowel suspended in Ringer's solution.

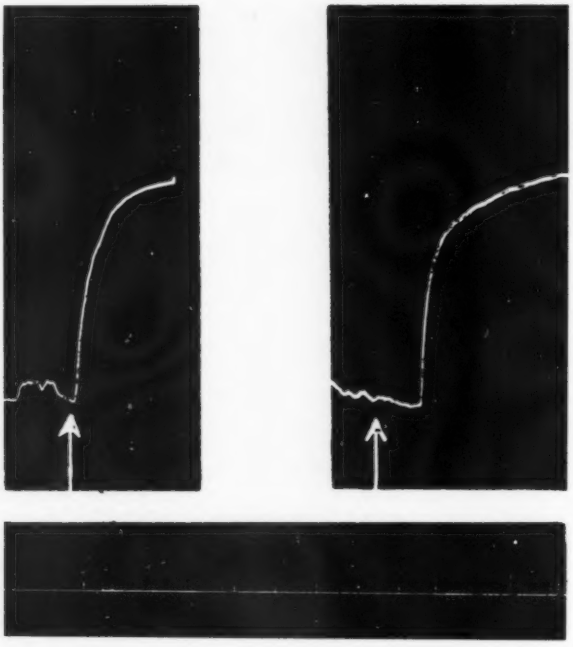

Fig. 10. Graphic record of contraction of isolated virgin guineapig uterus. In the left panel the response to 0·01 mg. histamine and in the right that to 10 mg. of platypus venom. Time in minutes.

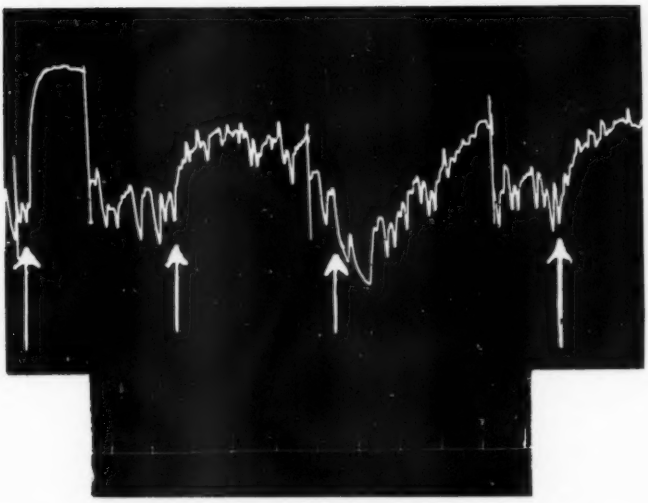

Fig. 11. Graphic record of contractions of isolated virgin guineapig uterus. From left to right at the arrows 0·01 mg. histamine, 0·002 mg. histamine, 2·0 mg. platypus venom, and 0·002 mg. histamine added to bath. Time in minutes.

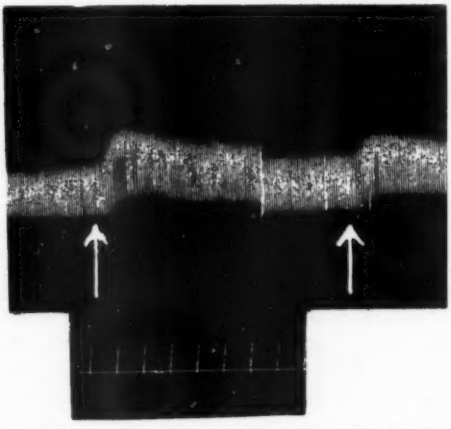

Fig. 12. Graphic record of contractions of isolated rabbit jejunum. At the first arrow 5·0 mg., and at the second 2·5 mg. platypus venom added to bath of 110 c.c. capacity. Time in minutes.

DISCUSSION.

Our observations have been limited by the amount of venom available, and are subject to confirmation by future experiments with fresh samples of venom. The examination of this old sample and of a small quantity of venom from a recently dead platypus gave results which are in accord with the symptoms caused in man by wounds with the spur of the male platypus.

Unlike the fresh venom examined by Martin and Tidswell, our old sample had but little coagulant power. A very feeble thrombin was probably present, as indicated by prolongation of the coagulation time of shed blood in normal animals following the intravenous injection of large doses of venom and also by Miss Williams's observations of the clotting of citrated plasma *in vitro*. The recently collected sample of venom was somewhat more potent in this respect, as judged by the presence of a little whipped-out fibrin in the heart of a rabbit which died after the intravenous injection of 50 mg per kg.

The venom is very feebly haemolytic, and possesses some cytolytic action as evidenced by the local lesions produced in animals by subcutaneous injection. The haemolytic and cytolytic activities of the venom need further investigation using freshly-collected material.

The most striking feature of the action of the venom is the immediate and profound fall of blood pressure which follows its injection intravenously. This occurs in the absence of any coagulation within the vessels, which would readily explain it by cessation of inflow of blood into the right heart, or blockage in the coronary, pulmonary, or portal circulations. The fall of blood pressure like that produced in animals by the intravenous injection of much smaller doses of non-coagulant Australian snake venoms is attended by vasodilatation. This is peripheral and not central in origin. In neither case is there any evidence that the carotid sinuses or vagi play any part in causing the fall of blood pressure, or that it is due to any direct enfeeblement of cardiac action.

The vasodepressant effect of platypus venom differs from that produced by the Australian snake venoms in that it may be elicited several times by successive intravenous doses, whereas the first injection of snake venom desensitizes the animal so that subsequent doses have no further vasodepressant action. In a single experiment with our freshly-collected platypus venom, this difference was not demonstrated, and there is some indication even with the old sample, both in the whole animal and in the isolated ear and mesentery preparations, that desensitization can be produced by the exhibition of repeated and large doses of venom. The venom also differs from snake venom in causing vasodilatation in the isolated rabbit's ear, whereas snake venoms cause vasoconstriction.

The condition of peripheral circulatory failure induced by the intravenous injection of large doses of platypus venom is prolonged and enhanced by basal and volatile anaesthetics.

Platypus venom is devoid of any peripheral curari-like effect, nor has it any specific action on the central nervous system. It has no direct effect upon respiration other than that resulting by carotid sinus reflexes set up by by the fall of blood pressure. Like Australian snake venoms it has a direct though much feebler stimulant action upon isolated smooth muscle, but it differs from these in that the initial response does not desensitize the muscle to second and later application of venom.

Martin and Tidswell have pointed out the general resemblance of platypus venom to snake venom. In the absence of a powerful neuro-toxic action and in the possession of coagulant, feebly haemolytic and cytolytic properties, it resembles the viperine venoms. The effects of a wound by the spur are also similar to but much less serious than those of envenomation by the bite of a viper, both in the local effects at the site of the wound or bite, and in the general manifestations due to peripheral circulatory failure.

SUMMARY.

The examination of an old sample of platypus venom and of one collected from a recently dead animal emphasizes its resemblance to a very feebly toxic viperine venom. It possesses weak coagulant, haemolytic, and cytolytic power. It has no specific neurotoxic action. Its main effect when injected intravenously is to cause a fall of blood pressure which is due to vasodilatation, and is peripheral and not central in origin.

ACKNOWLEDGMENTS.

Our thanks are due to Sir Charles Martin, whose generosity in giving us a sample of venom made this work possible; and to Mr. F. Lewis, Chief Inspector of the State Fisheries and Game Department, who gave us access to a recently dead platypus.

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THE ULTRA-VIOLET ABSORPTION SPECTRA OF SNAKE VENOMS

by

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(Submitted for publication 25th September, 1935.)

As specimens of the dried venoms of a number of species of snakes had been acquired by this Institute for the purposes of research on their physiological effects, it was decided to determine their absorption spectra for ultra-violet light in order to see whether any peculiarities that might be found could be correlated with their differences in toxic activity or with the genera to which the species belonged.

A Hilger medium spectrograph and rotating sector photometer were used for the research; the source of ultra-violet light was a spark between tungsten steel electrodes. The venoms were dissolved in a little very dilute sodium hydroxide solution, and the solutions diluted with water to a known volume. They were then centrifuged to remove any slight trace of suspended matter. The final concentration of alkali was in general about 0·0005N. Most of the venoms studied showed a small absorption band in the region of 280$\mu\mu$, but some had a band at a somewhat shorter wave-length.

The venoms used included specimens from the commoner Australian snakes, from several species of rattlesnakes and vipers, and from several Indian snakes. The Indian venoms came from the collection of Major George Lamb, presented by Sir Charles Martin. The North and Central American venoms were given by Dr. Thomas Githens, of the Mulford Biological Laboratories, and by Dr. Ledingham, Director of the Lister Institute. The milking from a single specimen of *Agkistrodon piscivorus* was given by Dr. Raymond L. Ditmars, and those of European vipers were provided by Dr. Schlossberger, of the Reichsgesundheitsamt, Berlin. In a number of cases more than one specimen of the venom concerned was examined. Different specimens of the venom of one species showed small differences in their absorption curves. In the case of the puff adder *(Bitis arietans)*, one specimen showed a well-defined band while the other, though readily soluble, showed no band, only general absorption. The values for this snake are omitted in consequence of this discrepancy. The venom of the platypus *(Ornithorhyncus anatimus)* showed no band. It was, however, an old specimen. In the case of the venom of the tiger snake *(Notechis scutatus)* little difference was observed in the absorption curve,

whether the venom was dissolved in a dilute acid, water, or dilute alkali. The venoms from two variants of the tiger snake showed similar absorption curves to that of the normal snake. They were (1) from a brown specimen, and (2) from a snake whose venom was quite white instead of the usual pale lemon colour. A third specimen, described as from a tiger snake entirely of a copper colour, had a very high absorption, with a well-defined band. Intravenous injections in rabbits showed, however, that it possessed the coagulant activity of the normal tiger snake venom.

The frequencies of the maximal and minimal absorption, together with the specific absorption coefficients at these frequencies will be found in the table. A few typical curves are shown in the graphs.

In general the absorption curves throw no light on differences in toxicity of the various venoms employed. The difference in toxicity of the two kraits is not reflected in any difference in the absorption spectra, and similar absorption occurs in both colubridae and viperidae.

The authors desire to acknowledge the generous loan of the spectrograph and photometer by Professor W. A. Osborne, of the University of Melbourne. One of them (H.F.H.) is indebted to the Australian and New Zealand Association for the Advancement of Science for a grant towards the cost of the plates used.

SUMMARY.

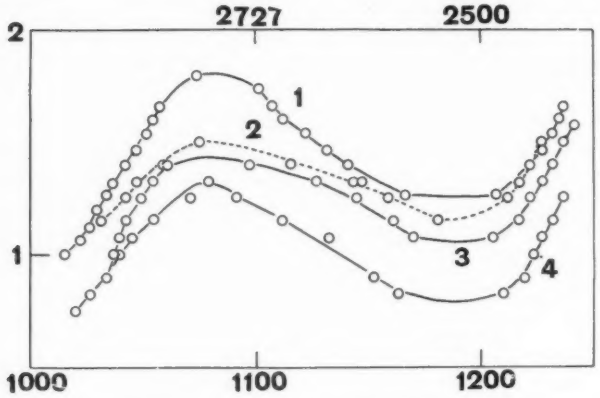
The ultra-violet absorption curves of a number of venoms have been determined. The curves are not correlated with known differences in toxic activity or with any biological classification of the snakes.

Absorption Coefficients for Positions of Maximal and Minimal Absorption by Snake Venoms.

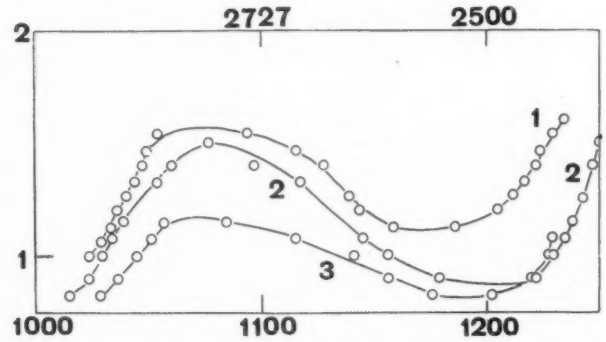
Species.		Maximum $\nu 10^{-12}$	ϵ	Minimum $\nu 10^{-12}$	ϵ
	VIPERIDAE				
North America					
Crotalus adamanteus	Diamond back rattlesnake	1073	1·8	1187	1·26
Crotalus atrox	Western diamond rattlesnake	1075	1·54	1180	1·12
" "		1083	1·4	1198	1·0
Crotalus confluentus confluentus	Prairie rattlesnake	1078	1·75	1189	1·15
Crotalus confluentus oreganus	Pacific rattlesnake	1088	2·07	1183	1·65
Crotalus exsul		1073	1·75	1186	1·15
Crotalus horridus	Timber rattlesnake	1079	1·5	1197	0·82
Agkistrodon piscivorus (single milking)	Water moccasin	1088	1·4	1195	1·075
" " (pooled sample)		1078	1·4	1187	1·075
Sistrurus catenatus	The Massasauga	1079	1·32	1186	0·82
Central and South America					
Bothrops atrox	"Fer-de-lance"	1075	1·5	1181	1·15
" "	" "	1075	1·5	1153	1·15
Bothrops neuwiedii	Jararaca	1090	1·2	1165	1·0
Crotalus terrificus	"Cascabel" tropical rattlesnake	1073	1·59	1180	1·38

Absorption Coefficients for Positions of Maximal and Minimal Absorption by Snake Venoms.

Species.		Maximum $\nu 10^{-12}$	ϵ	Minimum $\nu 10^{-12}$	ϵ
	VIPERIDAE				
India					
Vipera russellii	Daboia	1087	1·74	1183	1·4
" "	"	1101	1·57	1183	1·32
Echis carinata		1077	1·4	1181	1·07
" "		1085	1·68	1199	1·29
Europe					
Vipera berus	Common viper	1077	1·5	1199	0·9
Vipera berus, var. bosniensis	" "	1071	1·6	1182	1·2
Vipera ammodytes	Sand viper	1074	1·54	1172	1·12
Vipera lebetina	Blunt-nosed viper	1070	1·15	1189	0·82
	COLUBRIDAE				
India					
Enhydrina schistosa	Sea snake	1073	1·4	1115	1·32
Bungarus fasciatus	Banded krait	1087	1·82	1132	1·57
" "	" "	1107	1·8	1167	1·59
Bungarus caeruleus	Common krait	1094	1·82	1154	1·65
Naja naja	Cobra	1083	1·4	1147	1·15
Naja hannah	King cobra	1115	1·32	1196	1·2
" "	" "	1103	1·8	1190	1·54
Australia					
Notechis scutatus (acid)	Tiger snake	1132	2·24	1195	2·0
" " (water)	" "	1129	2·24	1198	2·12
" " (NaOH)	" "	1133	2·31	1198	2·1
" " brown variant	" "	1136	2·1	1200	1·68
" " unpigmented venom	" "	1117	2·1	1209	1·8
" " copper-coloured variant	" "	1154	3·47	1217	2·97
Notechis scutatus, var. niger	Black tiger snake	1117	1·82	1210	1·4
Oxyuranus scutellatus	The "taipan"	No band			
" "	" "	No band			
Denisonia superba	Copperhead	1132	3·0	1200	2·3
" " (mountain variant)	"	1132	3·1	1195	2·5
" " (melanotic variant)	"	1147	4·0	1217	3·5
Demansia textilis	Brown snake	1087	1·25	1155	1·0
" "	" "	1085	1·32	1172	1·12
Hoplocephalus bungaroides		No band			
Pseudechis porphyriacus	Black snake	1088	1·5	1186	1·15
" " (Queensland variety)	" "	1077	1·5	1186	1·07
Pseudechis australis		1101	1·9	1174	1·6
" "		1094	1·84	1183	1·44
Acanthophis antarcticus	Death adder	1143	3·15	1217	2·65
	MONOTREME				
Ornithorhynchus anatinus	Platypus	No band			



Graph 1. Curve 1, *Crotalus adamanteus*; 2, *Bothrops atrox*; 3, *Agkistrodon piscivorus*; 4, *Sistrurus catenatus.*

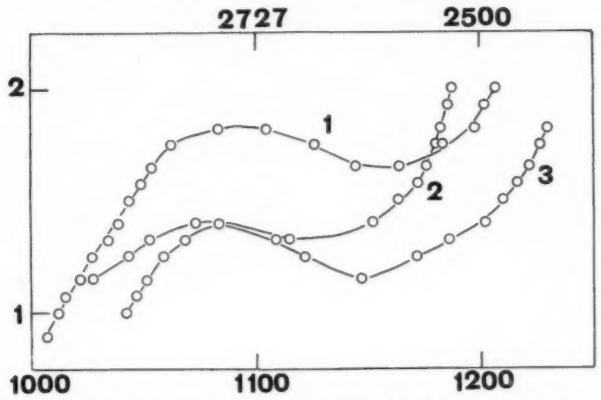


Graph 2. Curve 1, *Vipera ammodytes*; 2, *Vipera berus*; 3, *Vipera lebetina.*

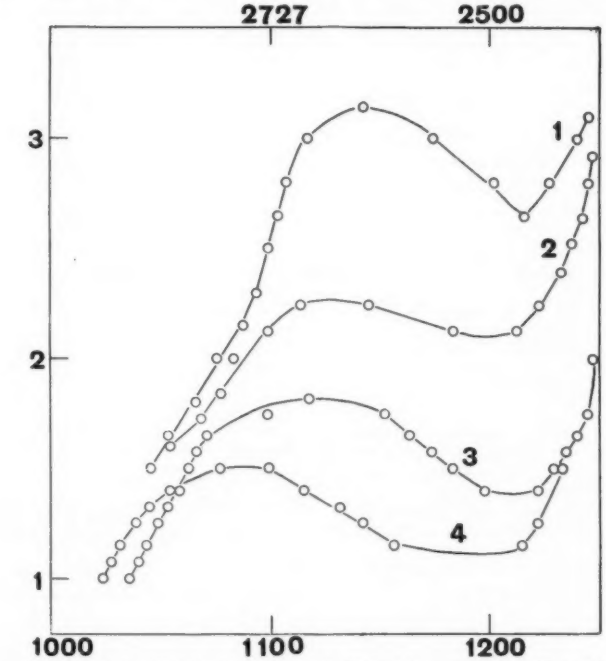
$$\epsilon = \frac{1}{cd} \log \frac{I_0}{I}$$

Where d = depth of liquid in cm.,
ϵ = number of grams in one litre,
I_0 = intensity of entering beam,
I = intensity of emergent beam.

Ordinates = ϵ
Abscissae = $\nu 10^{-12}$
Corresponding wave-lengths A°U. are added at the top of the graphs.



Graph 3. Curve 1, *Bungarus caeruleus*; 2, *Enhydrina schistosa*; 3, *Naja naja*.

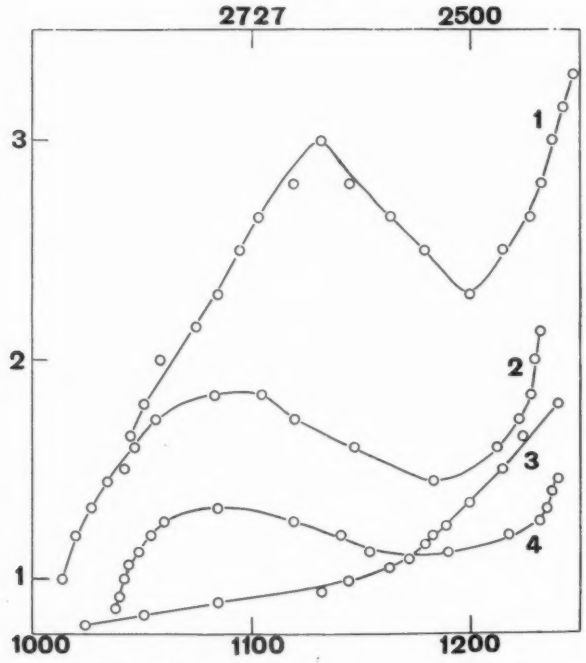


Graph 4. Curve 1, *Acanthophis antarcticus*; 2, *Notechis scutatus*; 3, *Notechis scutatus var. niger*; 4, *Pseudechis porphyriacus*.

$$\epsilon = \frac{1}{cd} \log \frac{I_0}{I}$$

Where d = depth of liquid in cm.,
c = number of grams in one litre,
I_0 = intensity of entering beam,
I = intensity of emergent beam.

Ordinates = ϵ
Abscissae = $\nu 10^{-12}$
Corresponding wave-lengths A°U. are added at the top of the graphs.



Graph 5. Curve 1, *Denisonia superba*; 2, *Pseudechis australis*; 3, *Oxyuranus scutellatus*; 4, *Demansia textilis*.

$$\epsilon = \frac{1}{cd} \log \frac{I_0}{I}$$

Where d = depth of liquid in cm.,
c = number of grams in one litre,
I_0 = intensity of entering beam,
I = intensity of emergent beam.

Ordinates = ϵ
Abscissae = $\nu 10^{-12}$
Corresponding wave-lengths A°U. are added at the top of the graphs.

OBSERVATIONS ON THE ESTIMATION OF CYSTINE BY SULLIVAN'S METHOD, AND A NOTE ON THE SUPPOSED CLEAVAGE OF CYSTINE FROM CASEIN BY DILUTE ALKALI

by

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(Submitted for publication 10th May, 1935.)

During recent years several colorimetric methods have been suggested which aim at the estimation of cystine in the solutions resulting from the hydrolysis of protein and food materials. Among these the method of Sullivan (1929) and its modifications (Prunty, 1933; Lugg, 1933) have been frequently employed owing to the apparent specificity of the reaction between the napthoquinone reagent and cystein in the presence of hydrosulphite.

As the reported analyses of fodder materials for cystine are not convincing, the analytical procedures have been subjected to critical test in this laboratory, and the findings indicate that these methods are capable of yielding misleading results.

Sullivan stressed that the napthoquinone reaction is specific for free cystine (or cystein), and although this has been recently questioned (White, 1934) it is usually deemed necessary when attempting to estimate the cystine content of a protein to ensure that the protein is completely hydrolysed.

Experiments planned to establish the relationship between the time of hydrolysis of casein and the amount of colour produced by Lugg's modification of Sullivan's reaction (apparent free cystine) led to inconclusive results. The disulphide content (see Table 1) is practically constant, but the apparent cystine content is highly variable. No satisfactory explanation can be offered for this other than the admission that Lugg's modification of Sullivan's method is not applicable to such material.

The findings of an experimental hydrolysis are set out in Table 1.

TABLE 1.

Time of refluxing in 5·5 N. HCl (hours)*.	Per cent. Cystine computed from Disulphide. (Lugg).	Per cent. Cystine. (Lugg-Sullivan).
0	·26	·20
4	·25	·16
8	·24	·17
12	·25	·16
24	·25	·13
48	·25	·20

* The casein was hydrolysed in constant boiling (5·5 normal) hydrochloric acid on a sand-bath, and the excess of HCl removed prior to analysis by evaporation on a water-bath at 100° C., a process which involved about 15 hours further heating. The reaction of the resulting syrup was adjusted to pH 2·5 by the addition of dilute NaOH, and the volume made up so that 100 ml. contained the hydrolysed material from 7·0 gm. of casein. The humins were allowed to settle, and the supernatant liquor was almost completely decolourized by shaking with 1 gm. of Norit carbon at room temperature, a treatment which resulted in no loss of the total disulphide as estimated by Lugg's modified Folin-Marenzi reaction (1932).

Previous work in this laboratory (Lee, unpublished) indicated that the estimation of pure cystine by Sullivan's method was adversely affected by the addition of casein hydrolysate to the solution, and the conclusion which may be drawn from further experiments suggests that the lack of control of the reaction may be the origin of serious error when Sullivan's method is utilized for the estimation of cystine in acid hydrolysates.

Prunty's modification, while overcoming some of the uncertainties of reduction which were inherent in Sullivan's method, is heir to the same shortcomings in that no attempt is made to control the reaction of the final solution in which the

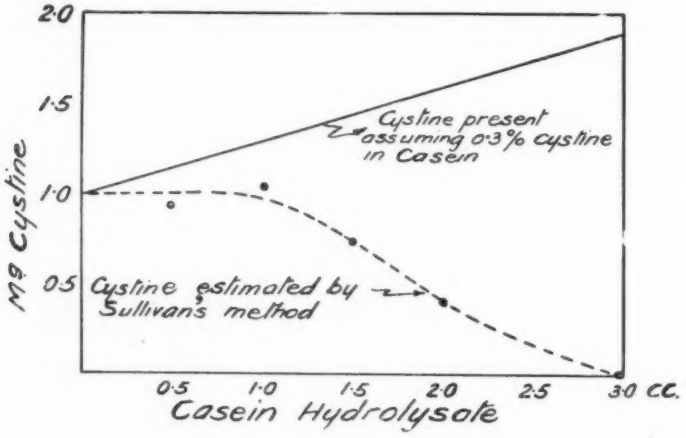

Fig. 1.

1 mg. cystine was added to each assay.

colour is developed. In our hands Prunty's modification of Sullivan's method proved not to be generally adaptable for the determination of small amounts of cystine in acid hydrolysates.

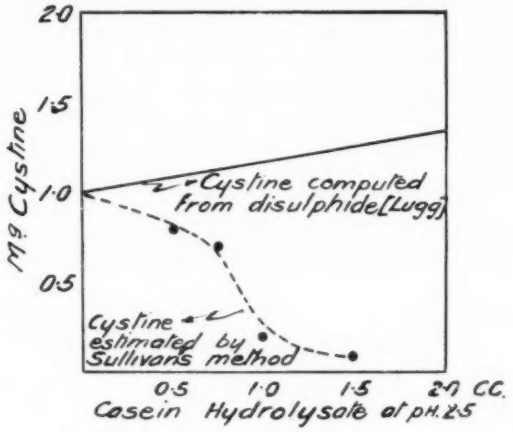

Fig. 2.

1 mg. cystine was added to each assay.

The results of typical experiments are recorded in Figures 1 and 2. In Fig. 1 the hydrolysate contained the mixed amino-acids arising from the hydrolysis of 10 gm. of casein in 100 ml. The solution was in 0·17 N acetic acid, but its pH was not determined. In Fig. 2 the hydrolysate was adjusted to pH 2·5. It contained 70 mg. of mixed amino-acids per ml., and the amount taken for the assay was made up to 5 ml. with 0·1 N HCl as stipulated by Sullivan. The pure cystine was in 0·1 N HCl solution; 1 mg. of pure cystine was added to each assay recorded in these figures.

It is evident from these data that increasing amounts of such hydrolysates have a marked effect on the colour produced in the Sullivan procedure.

To contrast the effects of buffered and unbuffered hydrolysates a solution of casein hydrolysate (48 hours in boiling 5·5 N HCl) was neutralized, and sufficient HCl added to make the whole to 0·1 N HCl (pH 5·0) as stipulated by Sullivan, and another for contrast was made to 0·01 N HCl (pH 6·6). For each assay the amount of hydrolysate taken was made to 5 ml. with 0·1 N HCl. These solutions contained 60 mg. of mixed amino-acids per ml. Cystine was estimated in varying amounts of these hydrolysates by Sullivan's method, and by Lugg's modification, in which the final solution is strongly buffered at pH 12·5.[1] The disulphide content was also estimated by Lugg's method.

The findings are graphically represented in Fig. III; and in Fig. IV the analyses have been repeated in the same solutions to which 1 mg. of cystine was added for each assay.

[1] Where necessary Lugg's specified volumes were increased to accommodate the amount of hydrolysate used without apparently impairing the efficiency of the method.

The effect of buffering the solutions is obvious, and if Lugg's method be employed the difficulties are largely overcome. The amount of cystine computed from such analyses, however, is always materially less than that suggested by the total disulphide content of the solution. The reason for this is at present obscure.

Fig. 5 graphically represents the analytical findings when a similar casein hydrolysate was used and the solutions buffered at pH 12·5. The variability of the analytical results is due to the difficulty in matching the final colours produced by the napthoquinone-sodium hydrosulphite reaction. Cystine standards appropriate to the amount of colour produced in the assays were used throughout.

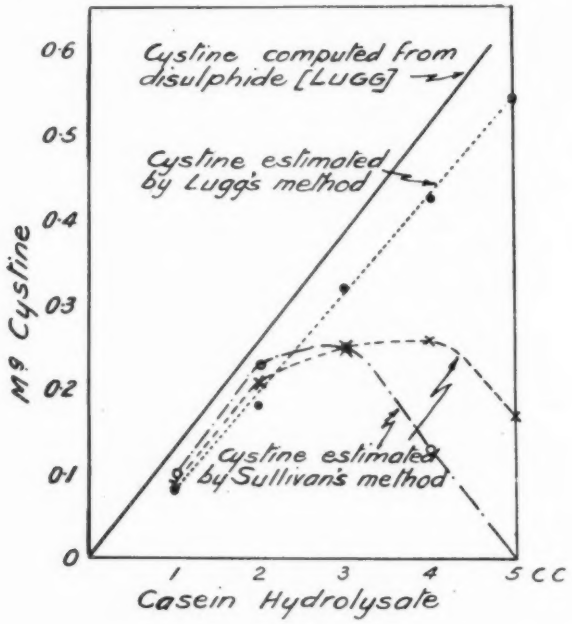

Fig. 3.

— · — · — Hydrolysate in 0·1 N HCl (pH 5·0).
— — — — Hydrolysate in 0·01 N HCl (pH 6·6).

In all methods utilizing the napthoquinone reagent a cloudy precipitate frequently appears in the final solution. Sullivan (1926) recorded that a white precipitate appears in casein hydrolysate during the procedure for estimating cystine, and stated (1929) that heavy metals interfere with the method by forming precipitates "especially on the addition of $Na_2S_2O_4$". Lugg made similar observations while utilizing his modification and recommended filtration to remove the cloudiness. The citrate in the buffer solution of the latter's reagent will prevent the precipitation of calcium phosphate, even when present in considerable quantities,

but it is not effective against precipitates of magnesium compounds which preliminary tests showed to be capable of producing characteristic precipitates.

The precipitate usually met with in Lugg's procedure is first discernible as minute flocculi that later settle out as a ruby-coloured precipitate, which, on exposure to air, turns blue and later fades completely. The precipitates produced by magnesium salts make their appearance immediately on adjusting the solution

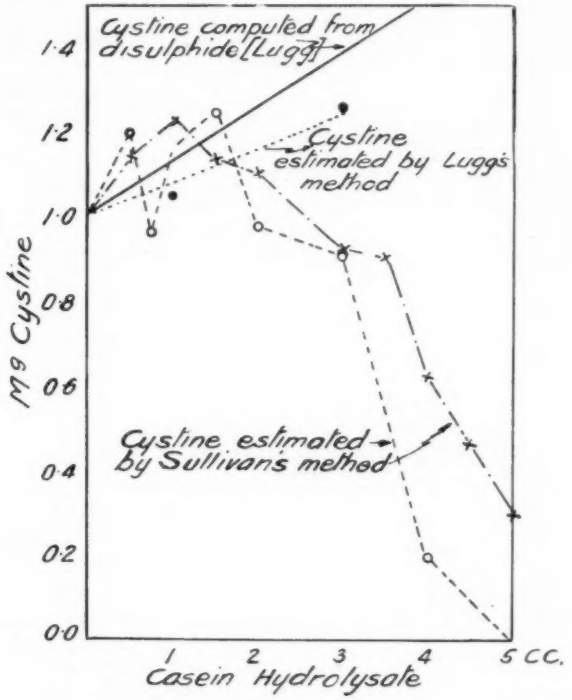

Fig. 4.

— — — — Hydrolysate in 0·1 N HCl (pH 5·0).
— · — · — Hydrolysate in 0·01 N HCl (pH 6·6).
1 mg. cystine was added to each assay.

to pH 12·5, but these are not readily seen until the dark colour of the excess reagent-amino-acid complex is discharged with sodium hydrosulphite. Such precipitates render accurate colorimetry impossible. The presence of magnesium as chloride in concentrations of 0·5 to 1·0 mg. in the final volume used for the estimation causes an overestimation of 1 mg. cystine by approximately 80 per cent., and 1 mg. of magnesium added to a cystine-free solution produces a precipitate whose colour, while in suspension, is roughly equivalent to that produced by 1·5 mg. cystine. In this last case the removal of the precipitate leaves a perfectly clear

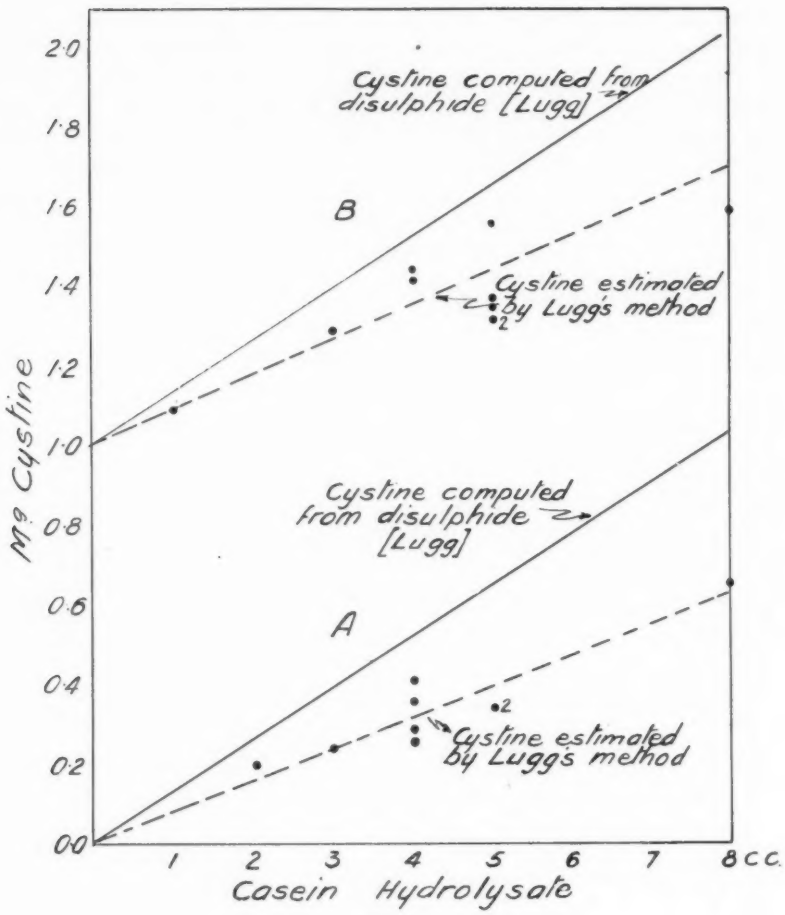

Fig. 5.

In B, 1 mg. cystine was added to each assay.

yellow solution. Difficulty was experienced in effecting complete removal of the precipitate by filtration, but this could be overcome satisfactorily by developing the colour in stoppered conical centrifuge tubes and centrifuging the final solution immediately after adding the hydrosulphite.

A loss of 13 per cent. of the colour produced by a hydrolysate of chick feathers containing 1 mg. of cystine was brought about by the removal of the precipitate from 1 mg. of magnesium by centrifuging the final solution; 0·5 mg. of magnesium under similar conditions involved an apparent 8 per cent. decrease.

Centrifuging the final solution could well be incorporated in Lugg's specified procedure, as in some cases the precipitate is not noticeable until the solution has been centrifuged.

It may be concluded that while removal of such a precipitate by this method may bring about underestimation of the cystine present, failure to remove it may result in very considerable overestimation.

The magenta colour which White (1934) claims to be produced by cystinyl-diglycine and cystinyl-dialanine with Sullivan's reaction is probably due to the contamination of these tripeptides with magnesium which was used in the course of their preparation.

On the Supposed Cleavage of Cystine from Casein by Dilute Alkali.

Jones and Gersdoff (1934) have recently published experiments which led them to conclude that the treatment of casein with dilute alkali results in the destruction of some of the cystine without affecting the other amino-acids. These authors based their conclusions on a series of analyses using Sullivan's procedure for the estimation of cystine.

From the experiences outlined above it would appear that Sullivan's procedure is not adequate for the estimation of the cystine content of the solution resulting from the acid hydrolysis of the alkali-treated casein. By modifying the analytical technique by carefully buffering (Lugg-Sullivan) the hydrolysate from casein that had been treated in a manner closely resembling that described by Jones and Gersdoff, we were unable to come to similar conclusions; and it would seem that these investigators were dealing with a similar phenomenon to that described in the previous section of this paper.

The procedure adopted is briefly: 40 gm. of casein (Harris) containing about 0·22 per cent. cystine in the air-dried material, moistened with 30 ml. of alcohol, were dissolved in 160 ml. of 0·1 N NaOH with mechanical stirring, 286 ml. of H_2O were added, together with 24 ml. of N NaOH, the temperature being 37° C. Solution was complete within one minute. Fifty ml. were withdrawn at once, and the main bulk was maintained at 37° C. for 24 hours, aliquots being drawn from time to time.

Each sample was immediately diluted with water, stirred mechanically, and sufficient 0·1 N acetic acid was added to bring the solution to pH 4·6. The precipitate was thoroughly washed with very dilute acetate buffer at pH 4·6, and then hydrolysed in boiling 5·5 N. HCl for 18 hours. Nitrogen, disulphide (Lugg) and cystine (Lugg-Sullivan) determinations were made on the hydrolysates. The findings are summarized in Table II.

TABLE 2.

Sample.	Time of treatment with alkali (hours).	Casein (N x 6·39).	Cystine from disulphide (Lugg).	Per cent.	Cystine by Lugg-Sullivan.	Per cent.
1	0	3·39 gm.	7·95 mg.	0·23	6·6 mg.	0·19
8	24	3·36 „	7·70 „	0·23	7·1 „	0·21

From these results it may be concluded that treatment of casein with alkali of the strength used by Jones and Gersdoff caused no apparent diminution of its cystine content. If one had conjectured that destruction would have occurred under such conditions and had attempted to overcome the difficulty of estimation of the supposed reduced amount of cystine by increasing the amount of hydrolysate used in the final assay of the unmodified Sullivan procedure, the results would have been similar to those in Fig. 1, and would have been sufficient to lead to the conclusion that cystine had actually been destroyed.

DISCUSSION.

The Sullivan method and any modifications in which no heed is taken of the final reaction of the colour solution are evidently capable of yielding erroneous results when attempts are made to utilize them for the estimation of cystine in protein hydrolysates containing small quantities of cystine. The few experiments reported above are sufficient to demonstrate that, if greater amounts of unbuffered hydrolysates are utilized in the analysis in anticipation of a low cystine content, the amount of red colour produced by Sullivan's procedure is *correspondingly smaller*. This fact is offered as an explanation of the results of Jones and Gersdoff's and of many other analyses where Sullivan's method has been employed. Even if the final solutions are carefully buffered the (Lugg-Sullivan) method in our hands presents many difficulties. The proportionality of the colour produced by small amounts of pure cystine is not good—for example, 0·4 and 0·2 mg. of pure cystine under identical conditions yield a colour ratio of 15 : 18. This lack of proportionality is due to the almost insurmountable difficulty of matching the solutions, for the interference of the residual yellow colour, which is not completely discharged by sodium hydrosulphite, is such as to render the method difficult to employ when assaying solutions containing relatively small amounts of cystine in the presence of considerable quantities of other amino-acids. Compensating colorimeters only partially overcome this difficulty. In casein hydrolysates the proportion of cystine to other amino-acids is considerably lower than 0·5 per cent. which Lugg recommends as the lower limit for his method.

SUMMARY.

1. Evidence is adduced to suggest that Sullivan's method is not suitable for the estimation of cystine in protein hydrolysates in which the proportion of cystine to other amino-acids is small.

2. Lugg's modification of Sullivan's procedure, in which the reaction is controlled by carefully buffering the solutions, is much more satisfactory.

3. Inherent shortcomings of the napthoquinone-hydrosulphite estimation for cystine are discussed.

4. On these grounds a possible explanation of the supposed preferential destruction of cystine in casein by dilute alkali reported by Jones and Gersdoff is offered.

ACKNOWLEDGMENTS.

I wish to record my thanks to Mr. H. R. Marston for suggesting these problems and for his help in the preparation of the manuscript, and to my colleague, Dr. J. W. H. Lugg, for much helpful advice during the work.

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MITOCHONDRIA, GOLGI APPARATUS AND VITAMINS

by

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(Submitted for publication 14th October, 1935.)

INTRODUCTION.

Giroud and Leblond (1934 and 1935) and the present author (1933 and 1935) have commented in previous papers upon the similarity of form which the vitamin C of the cell shows at times to that of the Golgi apparatus and mitochondria.

Although the relationship between these constituents is not yet quite clear, there appears little doubt that portion of the vitamin C of the cell is present in these cytological entities. It was thought possible that, in view of the fact that the vitamin C staining reagent on otherwise untreated tissue demonstrates only the reduced vitamin, a proportion of the vitamin C might exist in the mitochondria in the reversibly oxidized condition. Attempts have been made, therefore, to reduce any vitamin C existing in this condition before attempting to stain the tissue with the acid-silver nitrate solution.

Joyet Lavergne (1935) has shown by a series of cytological and cytochemical preparations that mitochondria contain vitamin A and glutathione. This has been confirmed by the present author.

The following work endeavours to show that vitamin C may be an essential constituent of mitochondria and of the Golgi apparatus, and that it is probably vitamin C, and not vitamin A (as suggested by Joyet Lavergne) that forms an oxidation reduction system with glutathione within the mitochondria.

As far as possible all tissues were examined in the fresh condition in order to avoid the possibility of artefacts due to fixatives and subsequent treatment.

Demonstration of Vitamin C.

Since vitamin C may occur in either the reduced or the reversibly oxidized form in the cell, it was found necessary to reduce all the oxidized vitamin present in order to obtain a picture of the entire vitamin content of the cell.

Two methods, one a modification of the other, were used. The first of these consisted in suspending thin pieces of fresh tissue in sulphuretted hydrogen for ten to fifteen minutes. The

sulphuretted hydrogen was then extracted by reduced pressure, and the tissue subjected to a stream of nitrogen for ten minutes. The tissue so treated was finally placed in the usual vitamin C staining reagent for one hour, fixed in photographic hypo, imbedded in wax, and sections cut in the usual manner. Other pieces of the tissue treated in this fashion were frozen, and sections 25μ thick were cut and dropped into the vitamin C staining reagent, being subsequently mounted and examined in glycerine. The second method consisted of the prior treatment of the tissue, for fixation purposes, in the vapour of glacial acetic acid.

Pieces of the same tissue untreated by the sulphuretted hydrogen method were subjected to the staining reagent with the object of comparing the reduced with the oxidized vitamin C of the cells.

Demonstration of Glutathione.

Frozen sections of various tissues were dropped into hot acetic acid (5 p.c.) for thirty to ninety seconds. The acetic acid was then drained away, and 5 p.c. sodium nitroprusside (saturated with ammonium sulphate) was then added and allowed to act for two minutes. Finally, a few drops of concentrated ammonium hydroxide were added, whereupon the sections turned a purplish blue.

Demonstration of Vitamin A.

In these preparations no attempt was made to differentiate between the provitamins and vitamin A itself. Anderson and Levine, however, state that at room temperature only vitamin A gives the Carr Price blue reaction, whereas at a temperature of 60° C. the carotenes give the reaction. It is probable, therefore, since all these preparations were made at room temperature, that only vitamin A was being demonstrated. Frozen sections were dropped directly into a solution of antimony trichloride in chloroform, and mounted in a drop of this solution. Immediate examination was made of these preparations, since the Carr Price blue colour fades very rapidly (within thirty minutes).

Demonstration of Mitochondria.

Mitochondria were demonstrated in fresh frozen sections by the use of diethylsafraninazodimethylanilin (Janus green B).

Golgi Preparations.

Golgi preparations were made by the usual osmium tetroxide methods.

All the above tests were carried out on pieces of liver, kidney, adrenal, and ovary of normal guinea pigs and rats.

In guinea pigs which had been placed on a scorbutic diet, frozen sections of the aforementioned tissues were placed in Janus green B and the vitamin C reagent.

On one occasion a sample of human urine was obtained from a case of pyelitis, in which were contained a very large number of epithelial cells. Mitochondrial and vitamin C preparations were also made of these cells after they had been centrifuged and washed free from urine.

RESULTS.

The granules and rodlets appearing in the glutathione and vitamin A preparations coincided with the mitochondrial preparations, and the present author is therefore able to confirm Joyet Lavergne's work (1935) on this subject.

With vitamin C the results were not so clear-cut. Firstly, however, there was very little difference between those preparations in which vitamin C had been reduced by hydrogen sulphide and those in which the tissue had been treated with the staining reagent without previous reduction. Accordingly it appears that, once the tissue is removed from the body, at any rate, the major portion of the vitamin C of the cell is in the reduced condition.

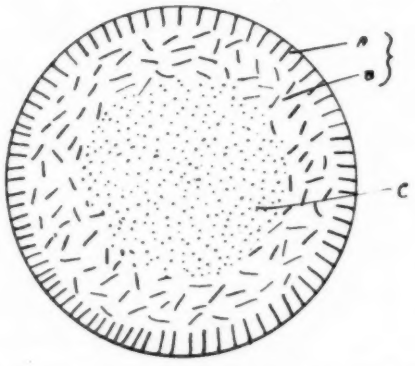


Fig. 1. Diagrammatic cross section through a single mitochondrium to show suggested structure: **A** and **B**. Lipoid-rich or water-poor phase of mitochondrium. **A.** Layer of molecules with long axes orientated in palisade fashion (Interface) (After Brailford Robertson). **B.** Lipoidal and fatty molecules of cortex scattered indiscriminately. No special orientation with respect to the surface. **C.** Water rich core of mitochondrium containing vitamin C, glutathione, and probably the proteolytic enzyme of Marston.

Secondly, in some of the vitamin C preparations the vitamin simulated the form of the Golgi apparatus; in others it simulated the mitochondria, and in some preparations both cytological constituents appeared to be imitated.

The obvious interpretation of these phenomena is that the cells of the tissues in the different preparations are in different chemical states.

A recent paper by Svirbely (1935) supplies a possible explanation of this problem. He shows that a certain concentration of glutathione will interfere with the silver nitrate test for vitamin C. It is possible, therefore, that vitamin C is normally present in both Golgi apparatus and mitochondria, but the occasional aberration of the staining reaction in the case of one or the other is due to the inhibition of the reaction by a certain concentration of glutathione in these constituents.

In scurvy, vitamin C appears to be withdrawn from both the mitochondria and Golgi apparatus. Apparently it is withdrawn from the Golgi apparatus first, because in the tissues of scorbutic guineapigs there was never any simulation of the apparatus after treatment by the vitamin C staining reagent.

The series of changes taking place in the cells of an organ such as the adrenal in progressive scurvy appear to be firstly a reduction in number of the granular elements; then follows a stage in which one still obtains a number of granules, but none of the cells show (in a great number of preparations) any simulation of the Golgi apparatus. Finally the granular elements practically disappear, although even in acute scurvy near the termination, some granules persist.

Mitochondria in Scurvy.

All these examinations were made on frozen sections of fresh tissue stained with Janus green B.

In the liver a number of the cells showed quite the usual amount of mitochondria. They were chiefly of a granular nature, however, no rods being observed.

In the red blood corpuscles three or four granules of normal appearance were observed.

In the adrenal cortex the number of mitochondria appeared to be reduced; a few rodlets were present, scattered between the lipoid droplets.

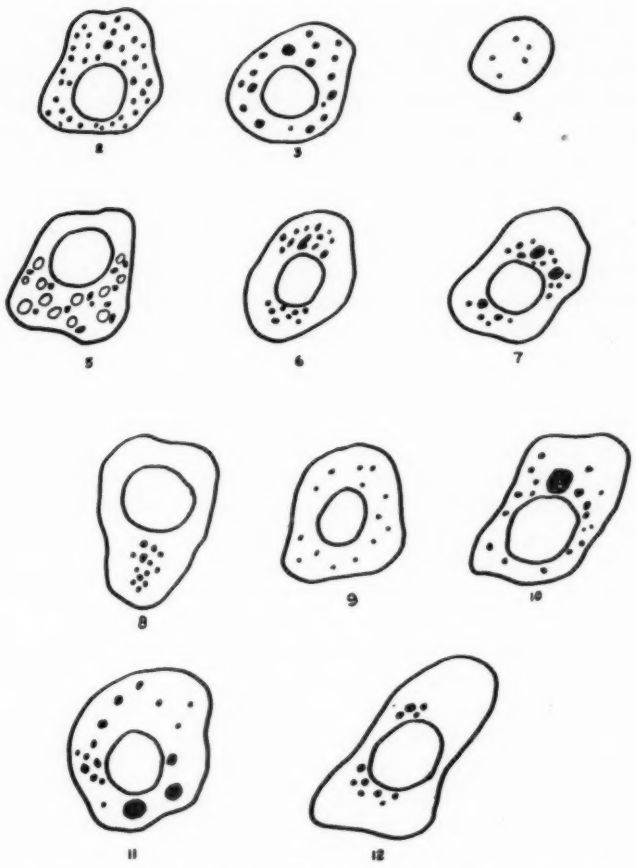
In the kidney the mitochondria varied considerably in shape and position. In some cells they were observed to be scattered in approximately equal numbers on either side of the nucleus. One group showed granules of equal size and the other granules of varying size and irregular shape; in some cells this inequality of size occurred in both groups of mitochondria, and in addition there appeared to have been a coalescence of some of the granules, for relatively large masses of mitochondrial material were observed. In other cells all the mitochondria appeared localized in one pole of the cell, and in others the granular mitochondria were scattered indiscriminately through the cytoplasm.

In a few cells of both kidney and liver there appeared to be a considerable degree of coalescence reminiscent to some extent of the condition in phosphorus poisoning; in some cells, masses of mitochondrial material were found a fifth of the size of the nucleus. That this condition was not due to the manipulation of the tissues was proved by its absence from normal tissues treated in an identical manner. In an occasional cell (from a scorbutic animal) the mitochondrial material appeared to be concentrated practically in one large globule.

The application of the vitamin C staining reagent to frozen sections from the same pieces of tissue showed that most of the cells contained only a few black granules—indicating that probably the vitamin C had been removed almost completely from the mitochondria.

Mitochondria in Pyelitis.

The mitochondria of washed epithelial cells from the human urine in a case of pyelitis were examined. The mitochondria were largely in the form of short filaments, although granular forms occurred also. The number of mitochondria appeared to vary greatly, as may be seen from the figures. In cases where the mitochondria were few they appeared to be chiefly granular.



Figs. 2–12. Mitochondria in scurvy. **Figs.** 2 and 3. Liver cells. **Fig. 4.** Red blood cell. **Fig. 5.** Adrenal cortex cell. **Figs. 6–12.** Kidney.

Vitamin C tests on the same material gave preparations which did not correspond with the mitochondria—there being very much fewer granules and rodlets. Vitamin C, however, is easily oxidized, and it is not known how long these cells had been exfoliated from the epithelial surface. Moreover, the cells were probably in a pathological condition owing to the infection of the urinary tract with *Bacillus coli communis*.

It has been shown, by the present author, that practically all cells of the body contain some granules of vitamin C, but it is known that all cells of the body contain a relatively large number of mitochondria; certainly a greater number of

mitochondria can be demonstrated in most cells than of vitamin C granules or rodlets; and biochemical estimation of the tissues for vitamin C shows relatively low concentration of the vitamin in all but a few organs. This is not what one would expect if all the mitochondria of every organ contained vitamin C. The conclusion is, therefore, that there are two forms of mitochondria—those containing vitamin C and those without vitamin C. In organs such as the corpus luteum, the anterior pituitary gland, the adrenal and the interstitial cells of the gonads where the mitochondrial and vitamin C preparations of the cell closely coincide, it is suggested that all the mitochondria contain vitamin C. It is also suggested, in view of the experimental work given in this paper, that the Golgi apparatus of cells invariably contains a certain amount of vitamin C, although it may not always be demonstrable, perhaps owing to an inhibiting concentration of glutathione.

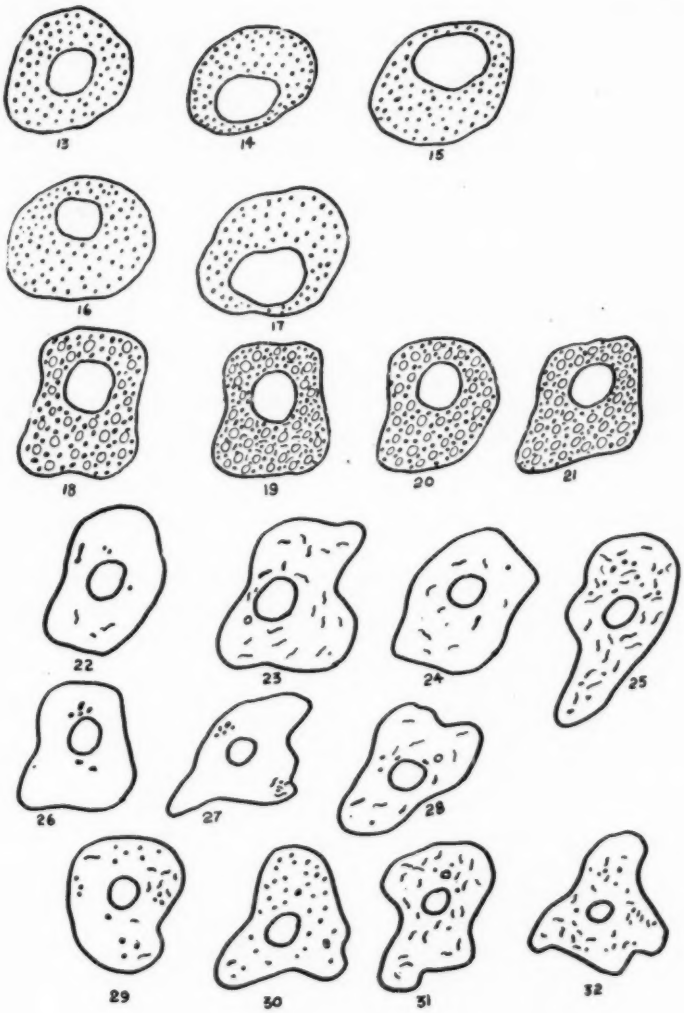
DISCUSSION.

The tendency of mitochondria to reduce osmium tetroxide, and their solubility in alcohol, ether, and chloroform, indicate a phospholipid or fatty nature; but the negative colour with Sudan III makes the latter unlikely. According to Cowdry they contain no polysaccharides nor any iron in protein combination.

In view of the fact that practically all mitochondria appear to contain vitamin A, Gatenby's (1919) observation of mitochondria in Limnoea, which were impregnated with a yellow pigment (almost certainly a carotenoid—a precursor of vitamin A) is of considerable interest.

As far as can be assumed at the present state of our knowledge, therefore, it appears that all mitochondria contain vitamin A and/or carotenoid pigments, glutathione, lipoidal substances, and probably also a protein, although neither Berg (1920) nor Noel (1923) were able to get a definite reaction on the part of mitochondria to Millon's reagent. In addition Marston (1923) considers that, because of the reactions of mitochondria with azin dyes, the former contain a proteolytic enzyme. The present work suggests also that some mitochondria at least contain vitamin C. In plants it is of interest that plastids are believed to be transformed mitochondria, and that Giroud and Leblond (1935) have shown that plastids reduce the vitamin C reagent.

Brailsford Robertson has pointed out that the molecules of the lipoidal material of the mitochondria which will, owing to the operation of the Gibbs Thompson law, be aggregated at the surface of the mitochondria, will be orientated in palisade formation, that is with the long axes of their carbon chains perpendicular to the mitochondrial surface. He showed that in mitochondria, therefore, we will have two phases. Firstly a water poor phase (the lipoid phase) at the outer portion of the mitochondria, and secondly a water rich phase in the centre of the mitochondria. In the former the vitamin A and carotenoids being fat soluble will be aggregated; in the latter glutathione, vitamin C, and the proteolytic enzyme are most likely to be concentrated.



Figs. 13, 14, and 15. Mitochondria (Janus green B) in cells of the pituitary gland. **Figs. 16 and 17.** Cells from sections of the same pituitary stained for vitamin C. **Figs. 18 and 19.** Cells of the adrenal cortex, showing mitochondria (Janus green B). **Figs. 20 and 21.** Cortical cells from sections of the same adrenal stained for vitamin C. **Figs. 22–32.** Showing the variety in the mitochondria in epithelial cells found in the urine of a case of human pyelitis. This variety is probably due to varying degrees of vitality of the cells as a result of the infection with *Bacillus coli communis.*

Joyet Lavergne (1935) points out that since vitamin A gradually absorbs oxygen and becomes oxidized it is, therefore, a reducing substance, and he postulates an oxidation reduction system formed of vitamin A and glutathione.

Apart from chemical considerations, however, the physical state of these two substances would make them unlikely as co-members of an oxidation reduction system. Vitamin A will be in the lipoid or water-poor phase, and glutathione will be aggregated in the water-rich phase. For this reason then it appears that Joyet Lavergne's conception of a redox system embodying a vitamin A-glutathione combination is not tenable.

In mitochondria which contain vitamin C it is suggested that it is the vitamin C which forms the "redox" system with glutathione, and not vitamin A. The combination of the first two in such a system is well-known in "*in vitro*" experiments, and one would naturally expect a similar association in regions of the cell where they may both be demonstrated.

The suggested structure of mitochondria is, therefore, shown in Figure 1.

The occurrence of vitamin A in the lipoid phase of the mitochondria is of considerable interest. The aggregation of the vitamin in this region is probably due to the selective solubility of the vitamin in the lipoidal substances at the surface of the mitochondria. The significance of vitamin A in mitochondria is not easy to assess. Very little is known as to the actual function of vitamin A in the body. Two of the most suggestive hypotheses so far are those of Jusatz (1933), who considers that it is associated with cellular permeability, and of Euler (1934 and 1935), who considers it to be associated with tissue oxidations. Vitamin A in the liver is believed by some workers to be associated with the system for oxidation of fatty acids there. It is possible that vitamin A may fulfil a similar function on the fatty materials of the mitochondria, in which case it will be associated also with variation of permeability, for this property of the mitochondrial membrane will vary as the chemical nature of molecules composing it are altered.

If we follow Regaud (1909) and assume an electosomic hypothesis for the function of mitochondria, i.e. the selecting of various substances from the protoplasm and their subsequent intramitochondrial elaboration, we must see that in view of the rather high concentration of lipoidal and fatty substances in the mitochondria, there will be a tendency towards a selective absorption of fat soluble substances. Vitamin A, if associated with permeability changes in the lipoidal cortex of the mitochondria, will facilitate or otherwise the passage of substances of varying degree of solubility in fatty materials into the interior.

Kingsbury (1912) has suggested that mitochondria function in protoplasmic respiration. It is considered that mitochondria which contain vitamin C are particularly well adapted to function as respiratory centres of the cell, and it may be seen that this type of mitochondria occurs in all cells of the body.

It is probable, therefore, that mitochondria have a complex structure, and the transformation theory of mitochondrial action, which is not supported by Cowdry, is rendered even more unlikely by the investigations recorded in this paper.

Cowdry mentions that the mitochondria are believed to consist chiefly of phospholipins in protein combination. The presence of a proteolytic enzyme in the mitochondria, as suggested by Marston's (1923) work, would provide a mechanism by means of which the protein portion of the compound molecule could be broken away leaving the lipoid free to be further changed if necessary, e.g. modification into fat drops. Horning (1929) in an excellent cytological study of mitochondria, suggests that "mitochondria are the loci of protein and possibly of general protoplasmic synthesis within the body of the living cell". The chemical nature of the mitochondria makes this suggestion quite feasible.

The Golgi Apparatus.

It is suggested from the results of this paper that vitamin C is contained in the Golgi apparatus of the cell. Some of the glutathione preparations have shown a structure similar to the Golgi apparatus also. To date, however, no structure resembling this apparatus has been found in the vitamin A preparations. It is possible that vitamin C and glutathione form an oxidation reduction system in the Golgi apparatus also, but the results so far are not sufficiently definite to make such a postulate desirable. All that can be stated with regard to this apparatus is that under some circumstances it appears to contain a certain amount of reduced vitamin C.

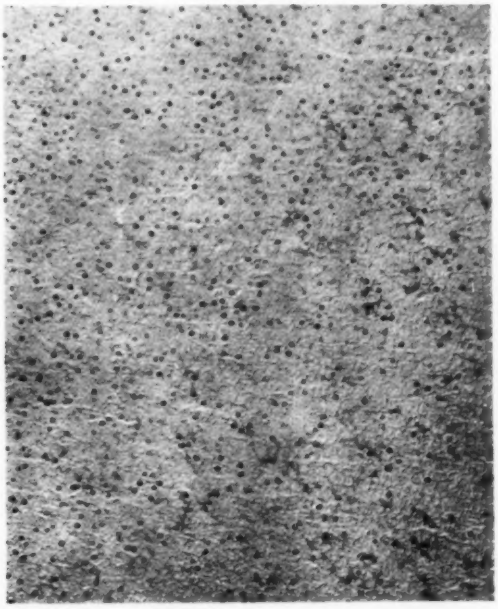


Fig. 33. Low power photograph showing impregnation of the Golgi zones of the cells by the vitamin C staining reagent.

SUMMARY.

Reduced vitamin C has been demonstrated in structures within the cells of various tissues which are believed to be the mitochondria and the Golgi apparatus. In addition Joyet Lavergne's work on the presence of glutathione and vitamin A in mitochondria has been confirmed.

It has been suggested that in view of the evidence adduced, the mitochondria may be divided into two groups—those containing vitamin C and those without it. Those mitochondria which contain vitamin C are believed to be ideally composed, chemically, to function as respiratory centres of the cell.

Mitochondria are suggested as being composed of an outer lipoidal cortex, which contain vitamin A and/or carotenoid pigments and a core (a water-rich phase) in which vitamin C and glutathione are situated—these substances forming an oxidation reduction system which is the basis of the activity of these mitochondria as respiratory centres.

ACKNOWLEDGMENT.

I am indebted to Mr. Russell Allen, M.Sc., Biochemist at this Institute, for his assistance in various phases of this work.

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THE FORMATION OF ACETYL CHOLINE IN THE BODY, AND AN INVESTIGATION OF THE CHOLINE ESTERASE

I, II, AND III.

by

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I.

While it may be claimed with almost certainty that acetyl choline (afterwards designated by A.C.) is the means by which the impulse is transmitted from the parasympathetic nerve endings to the particular organ, there is very little evidence to indicate the mechanism for the formation of this humoral substance.

Plattner (1933) found that if the tongue were divided and the lingual nerve to one-half stimulated, more A.C. was found in the stimulated than in the unstimulated half. Other workers (for literature see Vartiainen, 1934) also found an increased amount of A.C. in the heart and submaxilliary glands when their respective parasympathetic nerves were stimulated. More recently Beznak (1934) and Vartiainen (1934) have been unable to confirm the work of these earlier researchers.

On the other hand Geiger and Loewi (1922) found that the choline content of a gastrocnemius muscle increased when the sciatic nerve was stimulated. Kahlson (1933) holds that choline is formed from lecithin in the intestine, and its action is due to the further elaboration of A.C.

In view of these facts it was thought desirable to study the changes which bound and free choline may undergo in an organ when a parasympathetic nerve is stimulated. The organ chosen was the tongue because it could be divided into two symmetrical halves and the stimulated half compared with the control.

EXPERIMENTAL.

The method for the estimation of choline was similar to that devised by Roman (1930).

The neck of a dog was opened under chloroform anaesthesia, and the lingual nerve exposed; the circulation was stopped, and the tongue divided. The lingual nerve was stimulated for 20 minutes by a faradic current from a small induction coil. The stimulation was then stopped,

and the two halves of the tongue were removed and quickly sliced into 25 ml. of absolute alcohol in a beaker previously weighed, and the whole weighed again. They were then extracted for 20 hours, the alcohol poured off, and another 25 ml. were added and left for 4 hours. The two portions of alcohol were then combined, and evaporated to dryness on a water bath. The residue was then taken up in 10 ml. of water, and the lecithin removed by shaking three times with ether. The 10 ml. containing the choline were then dialysed through a collodion membrane into 200 ml. of water for 20 hours. The water was evaporated on a water bath to 10 ml., and the choline in 1 ml. determined. For the determination of the bound choline in lecithin, the ether washings were collected and evaporated to dryness. Then 20 ml. of a saturated methyl alcohol solution of barium hydroxide were added, and the whole heated on a boiling water bath under a reflux condenser for 2 hours. It was then acidified with 0·1 p.c. hydrochloric acid, filtered and evaporated to dryness. The choline was then taken up in 20 ml. of alcohol, the solution filtered and evaporated to dryness. It was then taken up in 10 ml. of water, dialysed, evaporated, and the choline estimated as before in 1 ml.

TABLE 1.

No. of Experiment.	Free Choline mg./100 gm. Stimulated.	Free Choline mg./100 gm. Control.	Bound Choline mg./100 gm. Stimulated.	Bound Choline mg./100 gm. Control.
1.	0·65	0·76	21·0	21·0
2.	0·90	1·00	9·9	10·4
3.	0·52	0·65	18·2	16·3
4.	0·80	0·90	15·0	17·0
5.	1·50	1·40	14·0	17·0
6.	0·50	0·40	19·5	15·0

It is evident that stimulation has not altered the amount of free or bound choline present within the limits of experimental error. And whatever may be the means of the production of A.C. it is not brought about by any alteration in the amount of choline present, either bound or free.

II.

One hypothesis put forward for the production of A.C. is that it is formed by the reverse action of the enzyme which brings about its destruction. That this synthesis can take place has been demonstrated by Ammon and Kwiatkowski (1934). An objection to this view has been raised by Henderson and Roepke (1933), who state that although it is well-known that A.C. is still formed in the body after the injection of eserine, yet this drug inhibits the action of the esterase on A.C. and should also inhibit the reverse action of the enzyme.

Accordingly it was deemed necessary to investigate the reverse action described by Ammon in the presence of eserine.

EXPERIMENTAL.

In a tube were placed 1 ml. of a solution of choline hydrochloride containing 0·5 gm. per 1 ml., 1 ml. of a 50 p.c. solution of sodium acetate, 1 ml. of pig serum, and 1 ml. of a solution containing sufficient eserine to bring the final concentration of eserine to 1 in 100,000, and finally one drop of

glacial acetic acid and a drop of toluene. A control was carried out, 1 ml. of water being added instead of eserine. Another control was attempted by endeavouring to destroy the esterase by subjecting a layer of serum 5 mm. thick to the influence of ultraviolet light (mercury arc) for 5 hours. Care was taken to add the eserine to the serum 20 minutes beforehand, as this has been found necessary to obtain complete inhibition of the enzyme. The tubes were then placed in the incubator at 40° C. for 20 hours. At the end of that time the amount of A.C. formed was estimated by means of a frog's rectus abdominus, both on the normal and that sensitized with eserine. Figure 1 shows some of the results obtained.

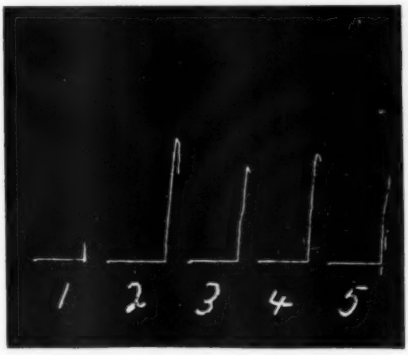

Fig. 1. Vertical lines indicate contraction on a stationary drum.

1. Choline, water, eserine (1: 100,000), serum.
2. „ Na acetate, eserine (1: 100,000), serum.
3. „ „ water, serum.
4. „ „ eserine (1: 1,000), serum.
5. „ „ water, irradiated serum.

From a consideration of Fig. 1 it will be seen that the synthetic action of the enzyme had not been affected by the addition of eserine. Further, in agreement with the findings of Galeher and Plattner (1927) ultraviolet light has scarcely any effect on this enzyme, because synthesis occurred equally as well with the irradiated serum.

Another possible explanation of this experiment is that eserine had combined with the A.C. formed and prevented its destruction. If this were so, then a higher concentration of eserine would allow the formation of more A.C., but as shown in Fig. 1 this is not the case. Further evidence against this view is supplied by the experiment, the results of which are shown in Fig. 2, where a concentration of eserine of 1 : 2,000,000 inhibited the destruction of all concentrations of A.C. employed. If eserine acted by combining with the A.C. then a definite concentration should prevent the destruction of a definite amount of A.C., but not of a higher concentration of the A.C.

From these facts one may conclude that the objection of Henderson and Roepke to the formation of A.C. in the body by the reverse action of the enzyme is not valid.

While no definite theoretical objection can be raised against the assumption that a substance could inhibit an enzyme action in one direction and not in another, it would make the point stronger if one could have support of this assumption. This will be attempted in the following section.

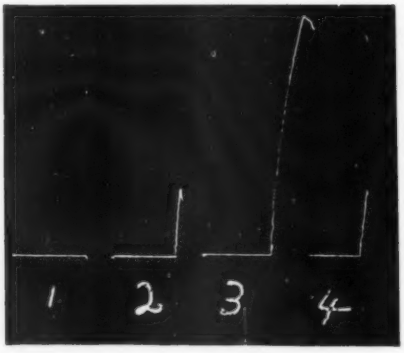

Fig. 2. Vertical lines indicate contraction on a stationary drum.

1. Serum, acetyl choline (1: 100,000), water.
2. „ „ „ (1: 100,000), eserine.
3. „ „ „ (1: 10,000), eserine.
4. „ „ „ (1: 100,000), pancreatin.

III.

Stedman (1932 a and b) has shown that the enzyme in serum which was capable of splitting A.C. was also capable of hydrolysing other esters, e.g. methyl butyrate and tributyrin. He has designated this enzyme choline esterase. In the last two cases the action was also stopped by eserine. The following experiments are designed to establish whether the synthesis of tributyrin by choline esterase is affected by the presence of eserine.

EXPERIMENTAL.

3 ml. of pig's serum were placed in a flask with varying dilutions of eserine, and after half an hour 5 ml. of a mixture of butyric acid and glycerine with only a little water added, and finally two drops of toluene. The flasks were placed in the incubator at 42° C. Twenty hours later they were removed, and 1 ml. taken from each, added to 5 ml. of alcohol, and titrated with N/10 NaOH. The difference from the control (water instead of serum) gave the amount of acid

which had been esterified. At the same time another control was carried out. Varying dilutions of eserine were added to 3 ml. of serum, and left to stand for half an hour. Then 10 ml. of a 1 p.c. ethyl butyrate solution were added, and the whole placed in the incubator at 42° C. for 20 hours. At the end of that time the flasks were removed, 20 ml. of water were added, and titrated with N/50 NaOH.

By this means one made definitely certain that the same concentration of eserine which inhibited the forward action allowed the reverse one to continue. Because there are at least two esterases in serum (see later), and because it is known (Galeher and Plattner, 1927) that choline esterase is comparatively heat-stable, higher temperatures than usual were employed to retard as much as possible the effect of the other esterases which are heat labile (Oppenheimer, 1925).

TABLE 2.

	No. of expt.	I	II	III	IV	
	Conc. of	Hydrol. of Eth. Butyrate		Synthesis of Tributyrin		Type of
Time.	eserine.	ml. N/50 NaOH.		ml. N/10 NaOH.		Serum.
0 hr.	Control	1·0	1·1	9·2	7·3	Pig
20 hrs.	„	5·6	4·3	9·0	7·0	„
„ „	$1/10^5$			9·0	6·7	„
„ „	$4/10^5$	2·6	2·8	8·5	6·4	„
„ „	$10/10^5$	2·5	2·7	8·4	6·4	„
	No. of expt.	V	VI	VII	VIII	
0 hr.	Control	1·0	1·0	6·9	4·6	Sheep
20 hrs.	„	6·0	7·0	6·7	4·5	„
„ „	$2/10^5$			6·7	4·4	„
„ „	$4/10^5$	3·5	4·0	6·3	4·2	„
„ „	$8/10^5$	3·4	4·0	6·0	4·1	„
„ „	$12/10^5$			6·0	4·1	„

The results of Table 2 on the hydrolysis of ethyl butyrate by pig or sheep serum indicate the presence of at least two esterases, one not affected by eserine and therefore similar to pancreatic lipase (see Table 4), and the other affected and therefore probably choline esterase. The results of the synthesis of tributyrin confirm those of the synthesis of A.C., namely that the synthesis can take place in the presence of eserine; but the concentration of eserine necessary is higher than that which completely inhibits the destructive action. The explanation may be that in the splitting of A.C. a low concentration of eserine, while not completely inhibiting the enzyme, does so to a large enough extent for the very minute amount of substrate present in that case.

Purification of Choline Esterase from Horse Serum.

The choline esterase was precipitated and purified according to the method of Stedman (1932b). The hydrolysis of ethyl butyrate in the presence of eserine was carried out by means of this purified preparation.

TABLE 3.

Time.	Conc. of Eserine.	ml. of N/50 NaOH. 1.	2.
0 hr.	Control	0·7	0·6
20 hrs.	Control	2·8	2·0
" "	$2/10^5$	2·7	2·0
" "	$5/10^5$	2·8	2·0

It is evident that eserine has not affected the action of this preparation.

Action of Eserine on Pancreatic Lipase.

Two drops of a 1: 500 solution of eserine were added to 2 ml. of a 4 p.c. pancreatin solution and allowed to stand; 20 ml. of a 1 p.c. ethyl butyrate solution were then added, and the whole placed in the incubator at 39° C. At the end of 2 hours it was removed, and titrated with N/50 NaOH.

TABLE 4.

Time.	Control. 1.	2.	Eserine treated. 1.	2.
0 hr.	1·2	1·1	1·1	1·1
2 hrs.	2·2	2·1	2·0	2·1

In agreement with Stedman (1932b) eserine has no effect on the action of pancreatic lipase.

The Action of Pancreatic Lipase on A.C.

1 ml. of a 4 p.c. pancreatin solution was added to 1 ml. of a 1 in 10,000 solution of A.C., and allowed to stand for several hours. When tested on the frog's rectus abdominus it was evident that no destruction had taken place. See Figure 2.

DISCUSSION.

From the results it is evident that eserine inhibits the destructive action of the enzyme present in pig and sheep serum which hydrolyses ethyl butyrate. The enzymes in the body capable of splitting this ester are the various lipases (pancreatic and liver) and choline esterase. That eserine is not harmful to the action of lipase has been shown by Stedman and confirmed by the author. Accordingly there is very good evidence that the enzyme which is destroying the ethyl butyrate is the choline esterase of Stedman.

Now to turn to the synthesis of an ester by means of this enzyme, Stedman has shown that choline esterase is capable of splitting tributyrin. Accordingly this synthesis has been chosen in preference to that of ethyl butyrate to obviate the danger of having ethyl alcohol present in the high concentrations necessary to demonstrate reverse action. From Table 2 it is evident that not only has the synthesis of tributyrin taken place in the presence of eserine (thus confirming the synthesis of A.C.), but with slightly higher concentrations of eserine an increased rate of synthesis was noticed. As far as can be ascertained no other case has been reported in which a substance inhibited the forward action of an enzyme but allowed the reverse one to proceed. And at first sight it may appear that there is

a theoretical objection to this assumption. But a little further thought will show that it is quite within the realms of possibility and, indeed, in itself has important theoretical bearings. If one considers the various theories of enzyme action, on one point alone are they in agreement, and that is that a union, either physical or chemical, takes place between the enzyme and the substrate. This union having taken place the substrate is then divided. Consider for a moment the hydrolysis of an ester according to this conception. The ester combines with some group on the enzyme and is then split into an acid and an alcohol. At the same time, because the reaction is dynamic and not static, acid and alcohol radicals must be combining with their respective groups on the enzyme complex, union takes place and an ester molecule leaves the enzyme. But, if into this system eserine is introduced it combines with the group on the enzyme with which normally the ester combines, and as this ester-enzyme combination cannot take place hydrolysis does not result; but as eserine does not affect the acid or alcohol group complexes, synthesis can still occur. Furthermore, because destruction has been inhibited synthesis should occur at an increased rate. That this is so is apparent from Table 2. Thermodynamical considerations are brought up by this fact, namely, if destruction is not taking place then synthesis should proceed past the point of equilibrium; but for this energy is necessary, and this an enzyme cannot supply. Whether the energy could be obtained elsewhere or complete synthesis occur is not dealt with here but in a later paper. Furthermore, this work was undertaken to determine the possibility of the reverse action of choline esterase being responsible for the elaboration of A.C. at the endings of parasympathetic nerves, not to elucidate the mechanism of enzyme action. The results obtained confirm the first possibility, but in the matter of enzyme action do no more than point the way along which further work may proceed.

The above results may be criticized on the grounds that the hydrogen ion concentration has not been controlled by the addition of buffers. This criticism is met by the statement that in all cases the results are comparative, i.e. the effect of eserine is compared with the effect without eserine, and accordingly any change in pH would affect each experiment to the same extent. That the addition of eserine did not significantly affect the pH was ascertained by determination of the pH before and after addition by means of the glass electrode. Further there would be a slight buffering action due to the serum present.

It would undoubtedly have been better to employ purified enzyme preparation in place of serum. And indeed Stedman claims to have purified the choline esterase from horse serum, but when his methods were applied to pig or sheep serum the preparations were very unsatisfactory. The enzyme was also prepared from horse serum, but unfortunately the action of this preparation on ethyl butyrate was not inhibited by eserine (see Table 3). It is remarkable that Stedman, having purified the enzyme, should not have tried the effect of eserine on its action. In all probability it is some other esterase in the preparation.

In another respect the results of Stedman (1933) could not be confirmed. Stedman was unable to find any choline esterase in pig and very little in sheep serum. Now Ammon has demonstrated that A.C. can be synthesized from choline and sodium acetate by means of the enzyme contained in pig serum. This has been confirmed by the author, and in addition he has been able to hydrolyze A.C. by pig serum (see Fig. 2).

With regard to the inability of pancreatic lipase to destroy A.C. the results are in accordance with those of Stedman (1932b).

Another interesting point arises in that it clarifies the ideas as to the types of esterase present in blood. That there is a fat-splitting enzyme in blood has been known from the beginning of the century. The origin of this enzyme, however, has not been settled. Hiruma (1913) and others postulated that it was derived from the pancreas. Prewitt (1923) was in favour of the view that it was a liver lipase. From Table 2 it is apparent that the esterase present in largest measure is choline esterase, but in addition there are one or more esterases which are not affected by eserine. With this fact in mind it is easy to realize the cause of the conflicting results of the early workers.

Finally, if the mechanism for the production of A.C. is the reverse action of the choline esterase, then one must interpret with caution some results obtained previously. One method of demonstrating that A.C. is liberated at the endings of parasympathetic nerves is to inject eserine and then stimulate the nerve. The underlying theory is that the eserine prevents the destruction of the formed A.C. which is allowed to accumulate and become detectable by our somewhat crude methods. In a great many cases, however, the mere injection of eserine itself is enough to mimic parasympathetic stimulation. Two explanations of this are possible (a) that eserine prevents the destruction of A.C. which is formed normally, and by its accumulation is measurable; (b) that the injection of eserine has upset the equilibrium between destruction and synthesis of A.C. in favour of an increased synthesis (this follows from the results of section III), and this plus the A.C. normally formed on stimulation is the amount of A.C. which is actually being measured. The objection to this view that the parasympathetic mimicking action of eserine is due to the production of A.C. by it, is the fact that, while the action of A.C. is abolished by atropine, it is generally accepted that that of eserine is not. But if one refers to the original paper (Winterberg, 1907) it is evident that small doses of eserine are annulled by atropine, and Winterberg himself states that only with certain limits did the action of eserine overcome that of atropine. Furthermore, while it is generally held that the action of A.C. is abolished by atropine, Barlow (1928) has shown that again this is true only within certain limits and larger doses of A.C. overcome the inhibiting effect of atropine.

More recently Feldberg (1934) has shown that if a section of intestine is perfused and eserine added to the perfusing liquid, A.C. appears in the perfusate. According to Feldberg there are two possible explanations. The eserine is either

inhibiting the tissue esterase or causing a secretion of A.C. He decides in favour of the former explanation. The experimental evidence brought forward proves that the eserine does inhibit the esterase, but this was quite early demonstrated by Loewi. However, bearing in mind this inhibiting effect, the results do not disprove the possibility of secretion. Indeed formation of A.C. must have taken place for it to be detected, even if it were protected from destruction. If the secretion hypothesis is disallowed, it follows that the A.C. extracted by perfusing with eserine-saline solution was present all the time, but this is absurd because it would have been hydrolysed immediately it was formed by the tissue esterase. Finally it is well-known that eserine acts on the endings of the parasympathetic nerves (but not after degeneration), and this is exactly where one would expect the liberation of A.C.

SUMMARY.

1. The bound or free choline content of the tongue is not altered by stimulation of the lingual nerve.

2. The synthesis of acetyl choline by pig serum is not affected by the presence of eserine.

3. The presence of at least two esterases in serum has been demonstrated. The action of one is inhibited by eserine; the action of the other is not.

4. The synthesis of tributyrin by choline esterase is not affected by eserine.

5. The esterase prepared from horse serum by the method of Stedman is not choline esterase.

6. The action of pancreatic lipase is not inhibited by eserine.

7. Pancreatic lipase does not hydrolyse acetyl choline.

8. Evidence is presented to show that the mechanism for the formation of A.C. in the body is by means of the reverse action of the tissue esterase.

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A NOTE ON THE COMPARISON OF THE ACTION OF CARDIAZOL AND CORAMIN ON THE RESPIRATORY CENTRE OF MAN

by

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In the course of animal experiment for class purposes, in 1933, it became evident that the relative doses of Coramin and Cardiazol necessary to produce the same effect on the respiratory centre, were such that the therapeutic margin with Cardiazol exceeded that of Coramin. Meanwhile, Behrens and Reichelt (1934) also working with rabbits, established the quantitative relations between the equally effective doses as being Coramin/Cardiazol: 8/1.

We then attempted to estimate more accurately the effect of these substances upon the respiratory centres of the human subject, for they are both important additions to the list of substances with definite stimulating effect on the respiratory mechanism. Coramin (CIBA) has the composition: 25 p.c. aqueous solution of pyridine-β-carbonic acid-diethyl-amide; whilst Cardiazol (Knoll) is: pentamethylenetetrazol.

Behrens and Reichelt had used the respiratory volume as an index of effect of the drugs, and we have measured rate, respiratory volume, and minute volume, using in all four subjects with good physique and large vital capacity. Whilst collecting these results we found that Steiniger and Gaubatz (1935a) had anticipated our work, which, however, we have completed as circumstances permitted. The results confirm the conclusions of these authors, who have quite recently (1935b) repeated the work using intravenous administration instead of subcutaneous injection.

The respiratory centre was first of all depressed by morphine, and then revived by the administration of the stimulant preparations, and continuous measurements of expiratory volume and rate were made. The average results have been expressed graphically, and indicate two facts of interest. There are two maxima in the minute volume curves separated by about two hours, and the response to Cardiazol is more rapid and extensive than that due to Coramin. Steiniger and Gaubatz showed in their experiments that Coramin had a depressing influence

upon the normal respiratory centre, whilst Cardiazol stimulated from the outset. We have been able to repeat this in only one case, which, however, gave almost identical results as those reported by the above workers. It does not appear necessary to use the intravenous route, as the response is obviously rapid enough for most purposes when the substances are administered subcutaneously.

SUMMARY.

1. The effect of Cardiazol on the human respiratory centre previously depressed by morphine, is more rapid and extensive than that produced by Coramin.

2. The results obtained by Steiniger and Gaubatz are entirely substantiated.

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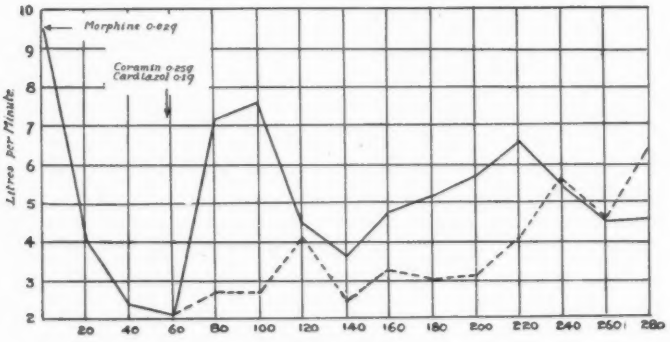
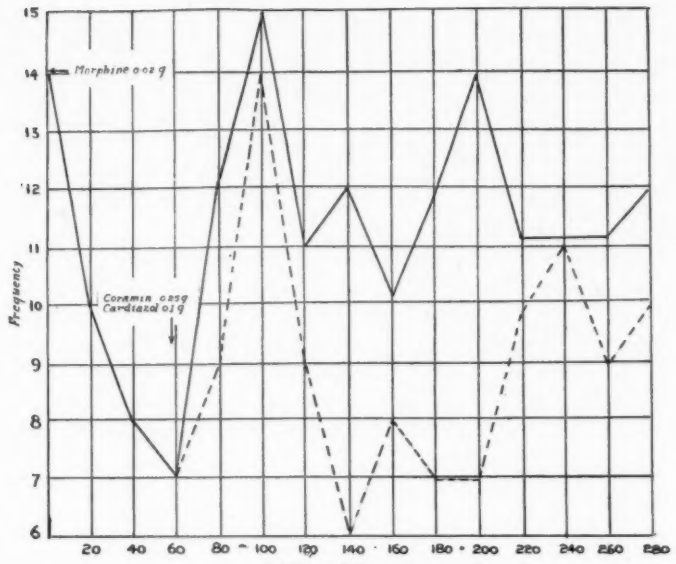
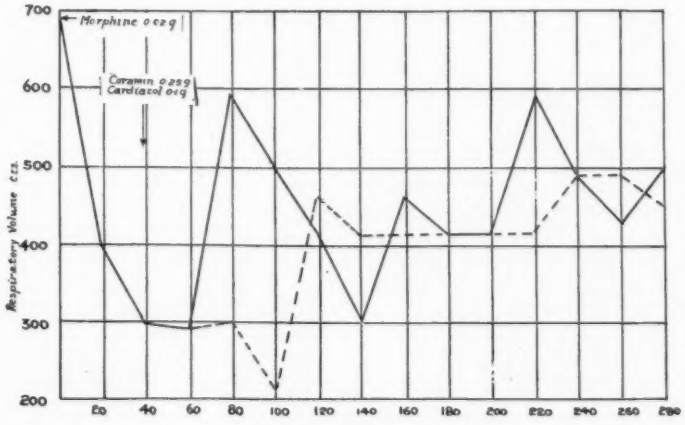


Time in Minutes.
Minute Volume. Cardiazol = Unbroken Line.

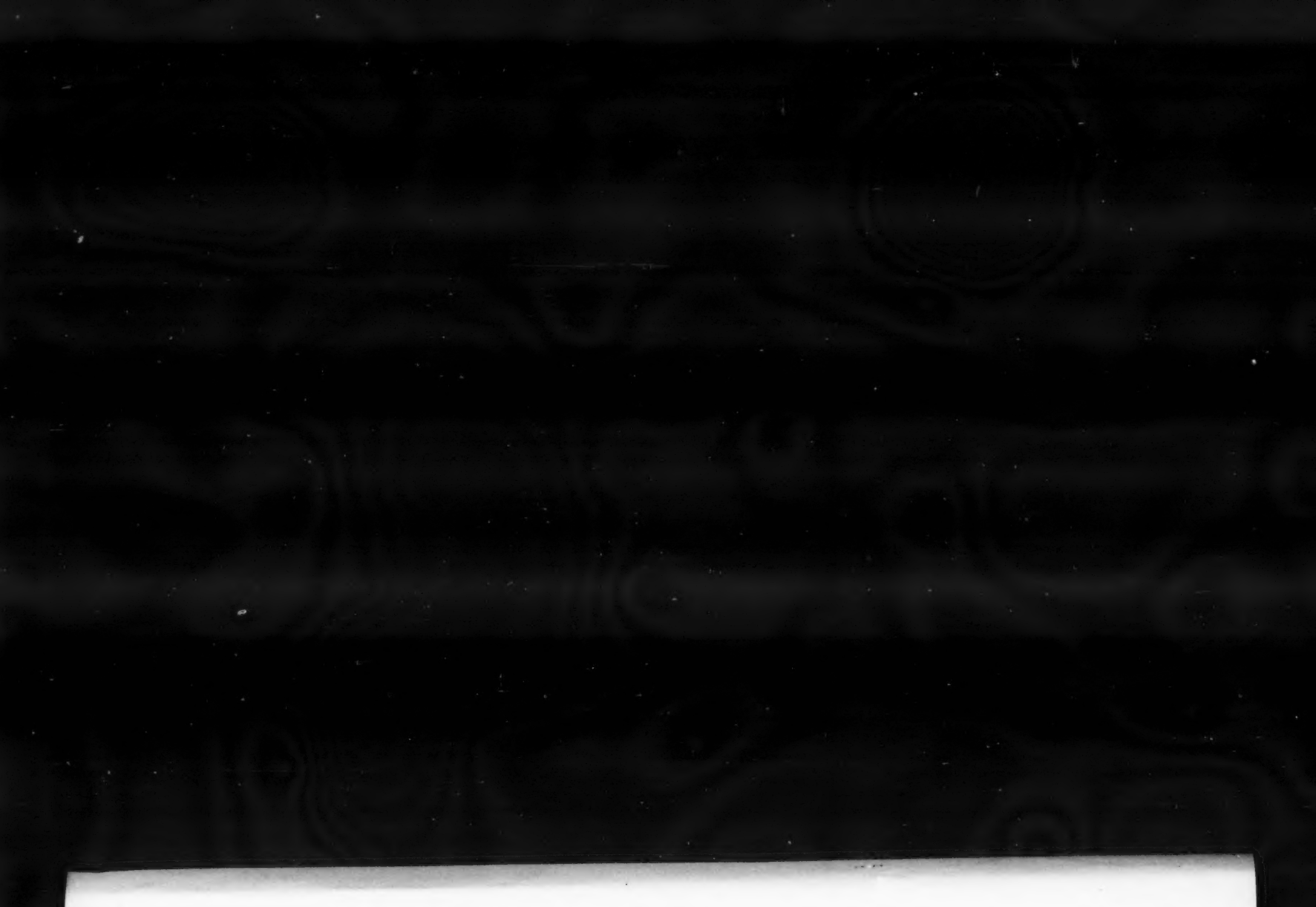


Time in Minutes.

Respiration Frequency. Cardiazol = Unbroken Line.



Time in Minutes.

Respiratory Volume. Cardiazol = Unbroken Line.

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